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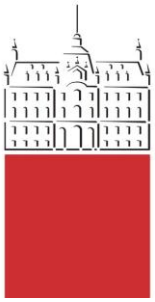

DOKTORSKA DISERTACIJA

Saša Haberl

Ljubljana, 2011

Univerza *v Ljubljani*
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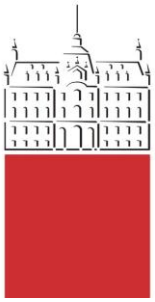

Saša Haberl

Analiza vpliva različnih parametrov na učinkovitost genske elektrotransfekcije v celičnih kulturah in v *in vitro* modelu tkiva

Analysis of the influence of various parameters on gene electrotransfer efficiency in cell cultures and in in vitro tissue model

Doktorska disertacija

Ljubljana, 2011

Mentorica: doc. dr. Mojca Pavlin

Somentorica: izr. prof. dr. Irena Mlinarič Raščan

Poročevalca: prof. dr. Julijana Kristl

prof. dr. Zoran Grubič

Izjava

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Celice so lahko zelo smešne. Le za malenkost jim
spremenimo gojišče ali ostale pogoje, v katerih rastejo,
in kot bi mignil, odmrejo. Lahko pa jih vzdražimo z
električnim poljem, ki je dovolj veliko, da bi ubilo konja,
in počele bodo tako neverjetne stvari, da znanstveniki o tem
razpravljajo na konferencah, pišejo obsežne knjige
in govorijo o čisto novi tehnologiji celične manipulacije.

Adrian Persegian

KAZALO

POVZETEK

Celična membrana je selektivno prepustni ovoj celice, ki omogoča nadzorovan pretok snovi. Z dovajanjem električnih pulzov dovolj visoke jakosti nastanejo na membrani celice začasne strukturne spremembe, zaradi katerih membrana postane prepustna za ione, molekule in makromolekule, ki drugače ne prehajajo skoznjo. Metodo vnosa molekul z električnimi pulzi so prvič uporabili leta 1972 in jo poimenovali elektroporacija. Danes je elektroporacija precej razširjena metoda, ki se uporablja v različne aplikativne namene, in sicer pri pripravi monoklonskih protiteles (elektrofuzija), inaktivaciji mikroorganizmov v tekočih prehranskih pripravkih (ireverzibilna elektroporacija), vnosu citostatičnih učinkovin v tumor (elektrokemoterapija), vnosu zdravil preko kože in transfekciji genov (genska elektrotransfekcija).

Uspešen vnos genov z elektroporacijo v celice *in vitro* je bil prvič opisan že leta 1982, za vnos genov *in vivo* pa se uporablja od leta 1991. Danes je genska elektrotransfekcija pogosto uporabljana raziskovalna metoda v predkliničnem in tudi kliničnem okolju, saj omogoča varen in preprost način vnosa genskega materiala. Kljub temu, da nam številne nevirusne metode prav tako omogočajo vnos genov v celice, so mnoge med njimi manj učinkovite, zapletene ali pa imajo neželene stranske učinke, ki so posledica vnosa kemičnih ali virusnih dodatkov. Natančni mehanizmi vnosa DNA v celico z dovajanjem električnih pulzov še vedno niso pojasnjeni. Znano pa je, da je genska elektrotransfekcija večstopenjski proces, ki zajame več korakov: vezavo DNA na membrano celice, prenos DNA skozi z električnimi pulzi permeabilizirane membrane (elektropermeabilizirana membrana), prenos DNA po citoplazmi celice, prenos DNA skozi jedrno ovojnico in izražanje vnesenega gena.

Na učinkovitost genske elektrotransfekcije vplivajo številni dejavniki. V literaturi lahko zasledimo vpliv dvovalentnih kationov, kot so magnezijevi ioni, na prvi korak genske elektrotransfekcije, in sicer na vezavo DNA na membrano celice. Ti pozitivno nabiti ioni naj bi namreč pripomogli k zmanjšanju elektrostatske odbojne sile med negativno nabito DNA in negativno nabito celično membrano in tako povečali uspešnost genske elektrotransfekcije.

Prvi del doktorske disertacije je osredotočen na vpliv magnezijevih (Mg^{2+}) ionov na elektropermeabilizacijo celične membrane, na uspešnost genske elektrotransfekcije in na preživetje celic po dovajanju električnih pulzov. Celice ovarijev kitajskega hrčka (CHO-K1) smo pripravili v suspenziji elektroporacijskih medijev z naraščajočo koncentracijo Mg^{2+} ionov. Dovajali smo električne pulze in kot indikator elektropermeabiliziranosti celične membrane uporabili molekulo propidijev jodid (PI), kot indikator uspešnosti genske elektrotransfekcije plazmidno DNA, ki nosi zapis za zeleni fluorescentni protein (GFP), preživetje celic pa smo določali z barvilom kristal vijolično. Naša študija je pokazala, da višje koncentracije Mg^{2+} ionov povečajo elektropermeabiliziranost celične membrane za majhne molekule in pripomorejo k boljšemu preživetju celic, medtem ko je uspešnost genske elektrotransfekcije znižana.

Vezava DNA na membrano celice po dovajanju električnih pulzov je lahko ob prisotnosti višjih koncentracij Mg^{2+} ionov tako intenzivna, da molekula DNA ne more prehajati skozi elektropermeabilizirano membrano. V nadaljevanju smo se zato posvetili vplivu Mg^{2+} ionov na prvi korak genske elektrotransfekcije, in sicer na vezavo DNA na membrano celice. Molekulo DNA smo obarvali s fluorescentnim barvilom TOTO-1 in dovedli električne pulze v prisotnosti 1 mM ali 50 mM koncentracije Mg^{2+} ionov. S fluorescentnim mikroskopom smo opazovali porast intenzitete fluorescence na tisti strani membrane, ki je obrnjena proti katodi. Porast fluorescence na tej strani membrane predstavlja obarvano DNA, nakopičeno na površini celice. Po dovajanju električnih pulzov v obrnjeni smeri smo opazili padec intenzitete fluorescence na celični membrani pri uporabi medija z 1 mM koncentracijo Mg^{2+} ionov, medtem ko tega ni bilo opaziti pri uporabi medija s 50 mM koncentracijo Mg^{2+} ionov. Zmanjšanje intenzitete fluorescence pri 1 mM koncentraciji Mg^{2+} in obrnjenem električnem polju povezujemo s šibkejšo intenziteto vezave DNA na membrano celice in posledično z lažjim prehodom skozi elektropermeabilizirano membrano in z večjo uspešnostjo genske elektrotransfekcije.

Ker naj bi dvovalentni kationi, predvsem Mg^{2+} povečali aktivnost encimov, ki razgradijo v celico vneseno DNA, smo preučili potencialni vpliv teh encimov na uspešnost genske elektrotransfekcije. Takoj po dovajanju električnih pulzov smo celicam CHO-K1 v suspenziji elektroporacijskega medija z 1 mM ali 50 mM koncentracijo Mg^{2+} dodali inhibitor DNaz, $ZnSO_4$. Pri dodajanju $ZnSO_4$ takoj po dovajanju električnih pulzov nismo opazili izboljšanja učinkovitosti genske

elektrotransfekcije, zato smo tezo o vplivu Mg^{2+} ionov na povečanje aktivnosti DNaznih encimov pri genski elektrotransfekciji ovrgli.

Drugi sklop doktorske disertacije je osredotočen na razvoj 3-D celičnega modela *in vitro* saj v bolj kompleksnih celičnih sistemih, kot so tkiva in organi, na učinkovitost genske elektrotransfekcije vplivajo tudi struktura tkiva, interakcija celic s komponentami zunajceličnega ogrodja in predvsem zmanjšana mobilnost DNA v zunajceličnem ogrodju. Analiza različnih parametrov genske elektrotransfekcije *in vitro* v okolju, podobnem *in vivo*, podaja možnost izboljšanja metode tudi v kliničnem okolju *in vivo*.

V doktorski nalogi so predstavljena različna tridimenzionalna (3-D) ogrodja, v katerih smo gojili različne celice in vanje skušali z dovajanjem električnih pulzov vnesti DNA. Za študijo genske elektrotransfekcije smo izbrali preprost 3-D kolagenski model, v katerem celice CHO-K1 preživijo, in smo vanje z dovajanjem električnih pulzov uspešno vnesli DNA.

Raziskovanje smo najprej usmerili v določanje primernih parametrov električnih pulzov za doseganje elektropermeabiliziranosti membrane tako gojenih celic. V nadaljevanju smo določali optimalne koncentracije DNA in optimalno izbiro parametrov električnih pulzov za uspešno gensko elektrotransfekcijo. Pokazali smo, da je v našem 3-D modelu dosežena najvišja transfekcija pri koncentraciji 90 μg/ml DNA v elektroporacijskem mediju in pri dovajanju serije osmih električnih pulzov, trajanja 5 ms, pri jakosti električnega polja 0.8 kV/cm, s ponavljalno frekvenco 1 Hz. Rezultati genske elektrotransfekcije v 3-D modelu so bili primerljivi z rezultati, pridobljenimi v okolju *in vivo*. Nadalje smo s kombinacijo visokonapetostnih (HV) in nizkonapetostnih (LV) električnih pulzov skušali izboljšati elektromobilnost DNA in s tem učinkovitost genske elektrotransfekcije v 3-D modelu. Analizirali smo tudi dovajanje različnih smeri električnih pulzov kot eno izmed možnosti izboljšanja metode *in vivo*. Dokazali smo, da z dovajanjem pulzov v različnih smereh izboljšamo uspešnost genske elektrotransfekcije v 3-D modelu.

Našo študijo smo v nadaljevanju usmerili v podrobnejše preučevanje mobilnosti DNA v 3-D modelu. V ta namen smo DNA nanesli bodisi na površino ali pa smo jo injicirali v notranjost 3-D modela in dokazali, da je uspešnost genske elektrotransfekcije

izboljšana pri vnosu DNA z injiciranjem, kar je bilo sicer tudi že pokazano s študijami *in vivo*.

Tretji sklop dela doktorske disertacije je usmerjen v transfekcijo DNA v primarne celice mišičnih mioblastov. Mišične celice predstavljajo pomembno ciljno tkivo za vnos terapevtskih genov *in vivo* zaradi dolgotrajnega izražanja vnesenih genov. Vnos DNA v tkivo *in vivo* omogočajo številne metode, ki pa se razlikujejo po svoji učinkovitosti. Zato je za optimalen vnos genov v okolju *in vivo* potrebna najprej analiza različnih metod vnosa genov v okolju *in vitro*. V raziskavi smo primerjali dva načina transfekcije DNA v primarne celice človeških mioblastov *in vitro*: gensko elektrotransfekcijo in lipofekcijo. Pokazali smo, da je učinkovitost obeh metod sicer primerljiva, da pa je lipofekcija manj citotoksična. Kljub temu v okolju *in vivo* bolj prednjači genska elektrotransfekcija, saj je metoda bolj preprosta in z njo ne vnašamo dodatnih kemičnih aditivov.

ABSTRACT

Cell membrane is selectively permeable cell envelope, which allows controlled transport of substances in and out of the cell. By applying short and intense electric pulses transient increase in the permeability of the cell membrane occurs for ions, molecules and macromolecules that otherwise cannot permeate through the cell membrane. A physical method for delivery of molecules by use of electric pulses (electroporation) was first described in 1972 and since then become used for different biomedical applications, such as preparation of monoclonal antibodies (electrofusion), food pasteurization (irreversible electroporation), introduction of poorly permeant anticancer drugs into tumors (electrochemotherapy), drug administration through the skin and gene transfection (gene electrotransfer).

Gene electrotransfer was first used in 1982 to introduce foreign genes into different cells *in vitro* and in 1991 into different tissues *in vivo*. Since then due to its safety and ease of application gene electrotransfer become used in a variety of preclinical and clinical settings. Although there are other non-viral methods that can be used to transfect genes into cells, many of them are less efficient, complex or can have different side effects, due to addition of viral or chemical factors. The processes underlying transfer of genetic material through cell membrane and into the cell by applying electric pulses are still not completely understood. Following steps are involved in gene electrotransfer: formation of a complex between DNA and cell membrane, translocation of DNA across the electropermeabilized membrane, transfer of DNA through the cytoplasm, translocation of DNA across the nuclear envelope and gene expression.

Gene electrotransfer efficiency is influenced by parameters, such as divalent cations, for example magnesium ions, which may have impact on the first step of gene electrotransfer, formation of a complex between DNA and cell membrane. Since DNA is negatively charged polyelectrolyte, magnesium (Mg^{2+}) ions can bridge the DNA with negatively charged cell membrane during application of electric pulses which leads to the improved gene electrotransfer efficiency.

In the scope of our study, we first have focused on the influence of Mg^{2+} ions on electropermeabilization of the cell membrane, gene electrotransfer efficiency and cell viability after applying electric pulses. Chinese hamster ovary cells (CHO-K1) were prepared in cell suspension in electroporation media with increasing concentration of Mg^{2+} ions. Electropermeabilization was evaluated with small molecule propidium iodide (PI) after application of electric pulses. Gene electrotransfer efficiency was evaluated with plasmid DNA that codes for green fluorescent protein (GFP) and cell viability with crystal violet dye elution method. Our study showed that higher concentration of Mg^{2+} ions leads to higher electropermeabilization for propidium iodide and higher viability, while causing lower gene electrotransfer efficiency.

To understand if the decrease in gene electrotransfer efficiency when using higher concentration of Mg^{2+} ions is a consequence of intense binding of DNA at cell membrane, visualization of DNA interaction with membrane was performed by using TOTO-1 labeled plasmid DNA. First train of pulses was applied in presence of different Mg^{2+} concentration (1 mM and 50 mM) to visualize complex between TOTO-1 labeled DNA and membrane. With fluorescent microscopy we observed the increase of fluorescence at the cell membrane facing the cathode, which can be explained by the accumulation of the labeled DNA at the cell membrane surface. After pulses of the opposite polarity were applied, decrease in fluorescence intensity in 1 mM Mg media was observed at the cathode side, but none or very small decrease in fluorescence intensity in 50 mM Mg media was detected. Our results therefore suggest that higher concentration of Mg^{2+} ions binds DNA at the cell membrane at such intensity, that DNA cannot cross the membrane during electroporation and gene electrotransfer efficiency is decreased.

It is known, that intracellular nucleases (DNases) can degrade plasmid DNA and that Mg^{2+} ions are necessary for enzymatic activity of DNases. Thus we studied if the efficiency of gene electrotransfer is affected by those enzymes. Immediately after pulse application (with 1 mM or 50 mM Mg^{2+} concentration), we added DNase inhibitor ($ZnSO_4$). Because we did not observe any improvement of gene electrotransfer efficiency by adding $ZnSO_4$, we suggest that Mg^{2+} ions do not have any significant impact on increased DNase activity in gene electrotransfer.

The second part of the doctoral dissertation is focused on the development of 3-D *in vitro* cell model. Namely, the main obstacle for *in vivo* gene electrotransfer still remains its efficiency since in tissues especially the diffusion of DNA is impaired due to extracellular matrix. There are also many other factors that influence the gene electrotransfer efficiency *in vivo*, such as tissue organization and cell interaction with extracellular matrix. For this reason analysis of different parameters of gene electrotransfer in an environment closer to *in vivo* conditions enables the possibility to optimize the protocol for more efficient clinical applications.

We therefore tested gene electrotransfer method in different three-dimensional (3-D) *in vitro* models. For further analysis, experiments were performed in a 3-D collagen model with embedded CHO-K1 cells, where successful gene electrotransfer was obtained and cells remained viable.

First we focused on determining appropriate pulse parameters for electropermeabilization of cells in 3-D model. Afterwards we determined optimal DNA concentration and pulse parameters to obtain successful gene electrotransfer. We showed, that highest gene electrotransfer efficiency was obtained at 90 μg/ml concentration of plasmid DNA in electroporation media and when a train of eight pulses with duration of 5 ms, electric field 0.8 kV/cm and repetition frequency of 1 Hz was applied. Gene electrotransfer efficiency in our 3-D model was comparable to the results obtained from studies *in vivo*. Furthermore we tried to improve electromobility of DNA in 3-D model in order to improve gene electrotransfer efficiency by applying combination of high-voltage (HV) and low-voltage (LV) pulses. Also pulses with different polarities were used to evaluate their role on gene transfer in 3-D model. Our results were consistent with previous results *in vivo* where gene transfection was increased when the electric field orientation between electrical pulses was changed.

We continued our studies with the aim to explore the mobility of DNA inside 3-D collagen model. For this purpose we compared gene electrotransfer efficiency for two cases: (i) electroporation media with DNA applied on top of 3-D model; (ii) electroporation media with DNA injected into a 3-D model. We observed that the latter way of DNA application showed higher transfection efficiency compared to the former one as was already shown also in *in vivo* studies, where DNA is usually delivered to the target cells by means of a local injection.

A third part of doctoral dissertation is focused on DNA transfection of human myoblasts. Muscle cells represent important target tissue for introduction of therapeutic genes *in vivo* due to prolonged expression of the introduced genes. Several methods allow DNA transfection into cells *in vivo*, but they differ in their effectiveness. First it is important to analyse different methods of gene delivery *in vitro* in order to optimize gene delivery *in vivo*. Therefore we compared two different methods for transfer of plasmid DNA in human myoblasts *in vitro*: gene electrotransfer and lipofection. We showed that efficiency of DNA transfection for both methods is comparable, while lipofection was less cytotoxic. Nevertheless, for some applications *in vivo* gene electrotransfer has advantage over lipofection due to simplicity of protocol and fact that no additional chemicals are used.

SEZNAM OKRAJŠAV

1306	humani kožni fibroblasti
2-D	dvodimenzionalno
3-D	tridimenzionalno
ADP	adenozin difosfat
ATP	adenozin trifosfat
B16F1	celice mišjega melanoma
CHO-K1	celice ovarijev kitajskega hrčka
CVDE	crystal violet dye elution method
DH5α	bakterijski sev *Escherichia coli*
DNA	deoksiribonukleinska kislina
DNaza	deoksirubonukleaza
ECM	zunajcelično ogrodje (angl. *extracellular matrix*)
EDTA	ethylenediaminetetraacetic acid
EGT	electrogene therapy
FBS	goveji serum (angl. *fetal bovine serum*)
FCS	telečji serum (angl. *fetal calf serum*)
G2 faza	del interfaze celičnega cikla
GFP	zeleni fluorescirajoči protein (angl. *green fluorescent protein*)
HV	visokonapetostni električni pulz (angl. *high-voltage pulse*)
IL-12	interlevkin 12
INF-α	interferon α
LV	nizkonapetostni električni pulz (angl. *low-voltage pulse*)
M faza	faza mitoze
OBP	orthagonal both polarity pulses
p53	tumor supresorski protein
PBS	fosfatni pufer (angl. *phosphate buffered saline*)
pEGFP-N1	plazmid, ki nosi zapis za zeleni fluorescirajoči protein
PI	propidijev jodid (angl. *propidium iodide*)
pIL-12	plazmid, ki nosi zapis za interlevkin 12
RNA	ribonukleinska kislina

SD	standard deviation
SP	single polarity pulse
TOTO-1	fluorescentno DNA barvilo
V79	pljučni fibroblasti kitajskega hrčka

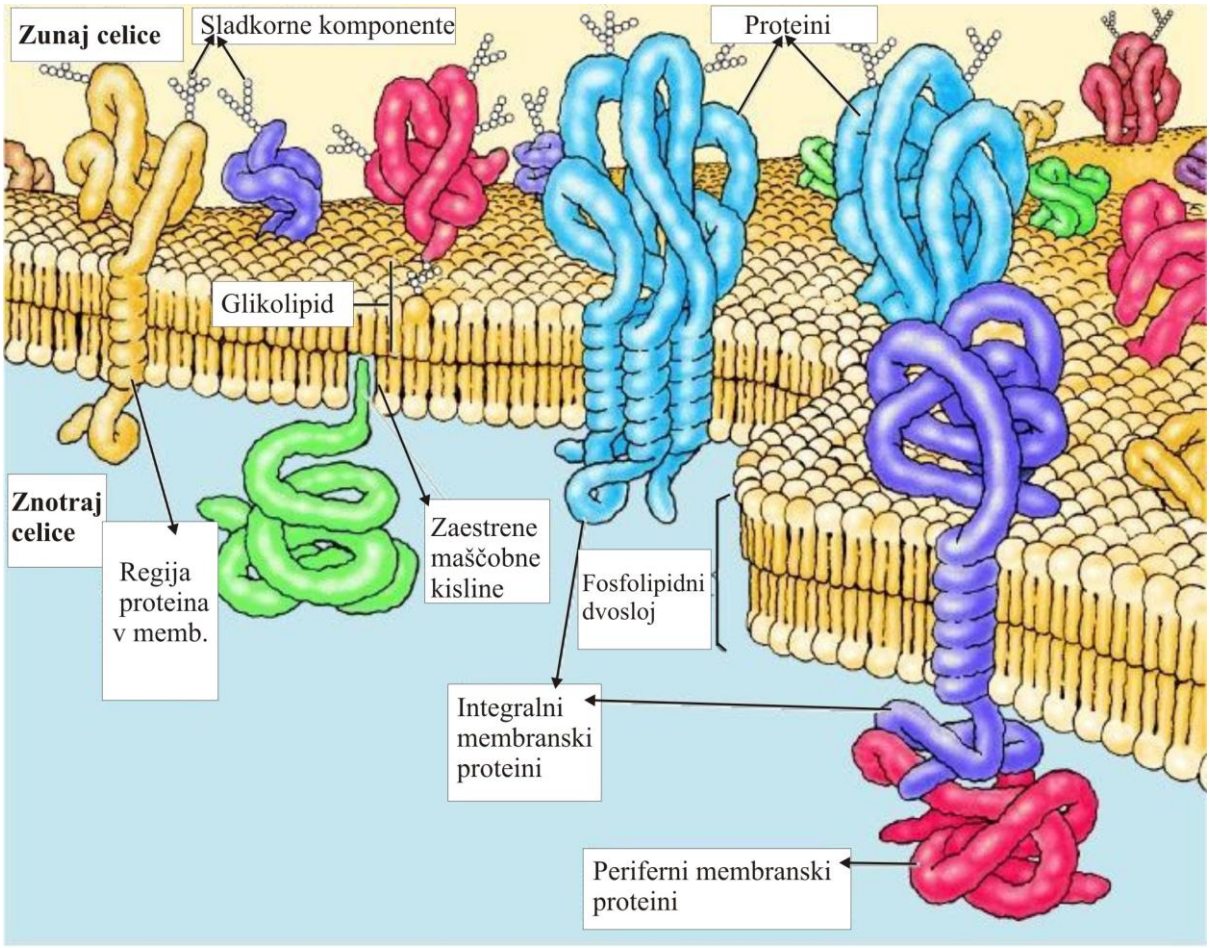
UVOD

1 CELIČNA MEMBRANA

1.1 Struktura celične membrane

Celica je strukturna in funkcionalna enota vseh živih organizmov. Znotrajcelična tekočina je ločena od zunajceličnega okolja s celično membrano (plazmalemo), ki omogoča selektiven prenos snovi, zagotavlja ohranjanje konstantnega notranjega okolja v celici in omogoča komunikacijo celice z okoljem (1). Plazmalemo sestavljajo trije tipi lipidov: fosfolipidi, glikolipidi in holesterol. Osnovno ogrodje predstavlja fosfolipidni dvosloj, sestavljen iz več vrst asimetrično razporejenih fosfolipidov (fosfatidilholina, fosfatidiletanolamina, fosfatidilserina, fosfatidilinozitola in sfingomielina). Na zunajcelični strani fosfolipidnega dvosloja se nahajata predvsem fosfatidilholin in sfingomielin, medtem ko se fosfatidiletanolamin in fosfatidilserin nahajata na znotrajcelični strani, kjer je v manjši meri zastopan tudi fosfatidilinozitol. Glikolipidi se nahajajo na zunajcelični strani in predstavljajo manjši delež vseh lipidnih membranskih molekul, medtem ko holesterol predstavlja večji delež membranskih lipidov in ga najdemo tako na zunajcelični kot na znotrajcelični strani fosfolipidnega dvosloja (2).

V osnovnem lipidnem ogrodju plazmaleme so asimetrično razporejene proteinske molekule (Slika 1). Ločimo integralne in periferne proteine. Integralni proteini lahko segajo skozi celotno plast fosfolipidnega dvosloja (transmembranski proteini) ali pa so vgrajeni v membrano le z lipidnim delom, na katerega je proteinski del vezan kovalentno. Periferni proteini pa niso vstavljeni v fosfolipidni dvosloj, temveč so povezani preko ionskih vezi z integralnimi membranskimi proteini.

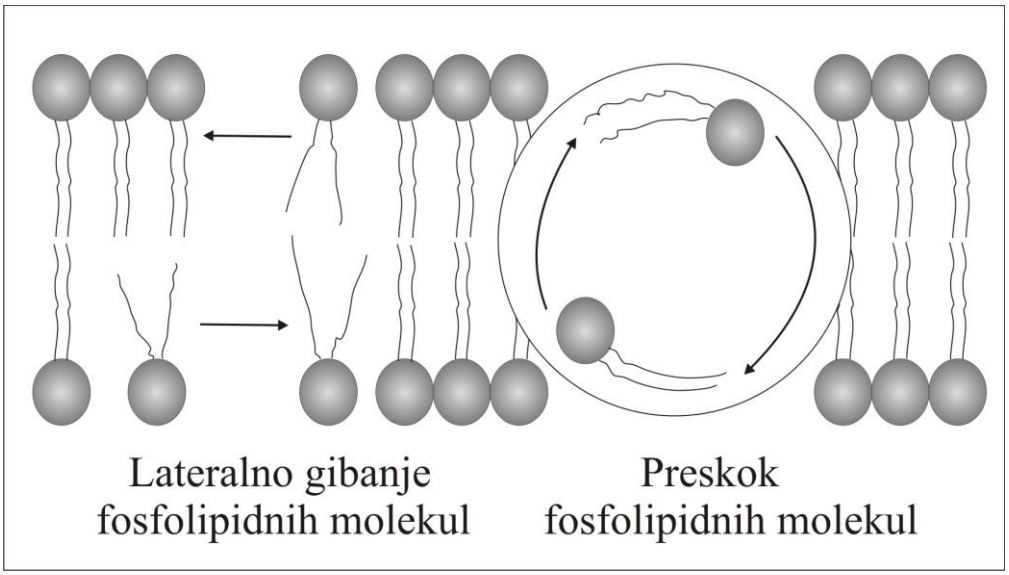


Slika 1: Shema celične membrane. Fosfolipidna dvoplast s proteini. Sliko smo povzeli po (2).

Fosfolipidne molekule v membrani so estri fosforjeve kisline. Alkoholna komponenta estra je glicerol, ki ima dve hidroksilni skupini zaestreni z maščobnima kislinama, eno pa s fosforjevo kislino, na katero je vezana različna organska baza. Molekule fosfolipidov so bipolarne, imajo polarni hidrofilni (glava) in nepolarni hidrofobni (rep) del. Glavo fosfolipida predstavlja glicerol s fosforjevo kislino in organsko bazo, medtem ko rep predstavljata maščobni kislini. Taka struktura jim omogoča značilno obnašanje v vodnem okolju, kjer se orientirajo tako, da je z vodnim okoljem v stiku le polarna glava (2).

Pomembna lastnost celične membrane je njena fluidnost. Fosfolipidne molekule so organizirane v dvodimenzionalno fluidno strukturo, v kateri se lahko posamezne molekule prosto vrtijo ali gibajo v lateralni smeri. Lateralno gibanje fosfolipidnih

komponent celične membrane je odvisno od komponent zunajceličnega ogrodja (3), temperature in vsebnosti posameznih lipidnih molekul. Predvsem holesterol pri višji temperaturi prispeva k večji rigidnosti membrane, medtem ko pri nižji temperaturi ohranja membrano v bolj fluidnem stanju (2). Poleg lateralnega gibanja pa je značilno tudi gibanje fosfolipidnih molekul iz enega sloja v drugega oziroma t. i. preskok (Slika 2). Tako gibanje omogočajo specifični encimi, flipaze, in je energetsko potratno (4).



Slika 2: Shematski prikaz gibanja fosfolipidnih molekul v celični membrani.

1.2 Transport skozi celično membrano

Celična membrana je selektivno permeabilna in omogoča nadzorovan pretok molekul v celico in iz nje. Majhne in nenabite molekule (O_2, H_2O, CO_2, N_2, glicerol, etanol itd.) lahko prehajajo membrano s pasivno difuzijo v smeri svoje nižje koncentracije, medtem ko večje molekule (glukoza, aminokisline itd.) ali nabiti ioni (H^+, Na^+, K^+, Ca^{2+}, Cl^- itd.) membrano lahko prehajajo le s pospešeno difuzijo ali aktivnim transportom. Pospešena difuzija poteka s pomočjo posebnih proteinov, ki jih glede na način prenosa molekul in ionov skozi membrano delimo na dve skupini:

a) Kanali: tvorijo odprto poro skozi fosfolipidno dvoplast in omogočajo prenos molekul primerne velikosti. Odprtje pore je selektivno in je stimulirano z zunajceličnim dražljajem (vezava primernega liganda, spremembe v električnem potencialu na membrani).

b) Prenašalni proteini: selektivno vežejo molekulo in s konformacijsko spremembo omogočijo njen prenos skozi membrano.

Aktivni transport praviloma omogoča prenos snovi v smeri nižje koncentracije proti višji. Tak prehod skozi membrano poteka s pomočjo posebnih struktur (molekulski izmenjevalci, prenašalci in črpalke) in zahteva energijo v obliki hidrolize energetske molekule adenozin trifosfata (ATP) (2).

Večje molekule, kot je deoksiribonukleinska kislina (DNA), pa celične membrane ne prehajajo.

2 GENSKA TERAPIJA

Genska terapija je način zdravljenja, pri katerem v celico vnesemo genski material z namenom nadomestiti, spremeniti ali utišati izražanje gena, ki predstavlja vzrok genske bolezni. Začetki genske terapije segajo v zgodnja sedemdeseta leta prejšnjega stoletja (5), prvi klinični preizkusi genske terapije pa so se začeli leta 1990 z zdravljenjem dedne bolezni hude kombinirane imunske pomanjkljivosti (6). Nadaljevali so se poskusi genske terapije na področju zdravljenja dednih recesivnih motenj, kot sta cistična fibroza (7, 8) ali Duchennejeva mišična distrofija (9) in danes je v teku že veliko predkliničnih in kliničnih študij genske terapije za zdravljenje poligenetskih bolezni, kot so diabetes (10), kardiovaskularna bolezen (11) ali nevrodegenerativna bolezen (12). Med kliničnimi študijami genske terapije pa je najbolj razširjena genska terapija za zdravljenje različnih malignih obolenj (13 - 16).

2.1 Metode vnosa genov

Glavni problem genske terapije še vedno predstavlja učinkovit in varen vnos terapevtskih genov do ciljnega tkiva. Danes poznamo različne načine vnosa genskega materiala, ki jih v splošnem delimo na virusne in nevirusne (17).

Virusni vnos: prednost uporabe virusnih vektorjev je predvsem njihova naravna sposobnost prenosa genske informacije v ciljno celico. Zato je za tak način vnosa terapevtskega gena značilna visoka specifičnost in učinkovitost ter stabilna vgraditev gena v celični genom. Za vnos terapevtskih genov uporabljamo retroviruse, lentiviruse, viruse *Herpes simplex* tipa 1 in adenosorodne viruse (17, 18). Še vedno pa se pojavlja vprašanje varnosti uporabe, zaradi možnosti nastanka insercijske mutageneze ali aktivacije gostiteljevega imunskega sistema (19).

Nevirusni vnosi: med nevirusne vnose štejemo vnos s pomočjo bakterij, vnos gole nukleinske kisline in vnos nukleinske kisline v lipidnem ovoju (lipofekcija).

a) Vnos genov z nepatogenimi bakterijami: za vnos terapevtskih genov uporabljamo bakterije rodu *Clostridium*, *Bifidobacterium* in tumor-invazivno bakterijo *Salmonella*. Prednost uporabe bakterij kot potencialnih nosilcev terapevtskih genov je večja varnost (zaradi poznane občutljivosti bakterij na antibiotike) in precej enostavna genska manipulacija (17).

b) Vnos gole nukleinske kisline: tako vnašamo plazmidno DNA, fragmente dvoverižne DNA in kratko interferenčno RNA (20). Prednost takšnega načina vnosa je večja varnost zaradi neprisotnosti dodatnih kemičnih ali potencialnih patogenih dodatkov, vendar pa je način manj učinkovit, ker serumske nukleaze hitro razgradijo nezaščiteno nukleinsko kislino (21).

c) Lipofekcija: da bi izboljšali stabilnost terapevtskega gena zunaj celice lahko nukleinsko kislino vnesemo v lipidni ovoj (22 - 24). Z vgradnjo specifičnih komponent v lipidni sloj (ligandov za specifični celični receptor ali protiteles proti specifičnim komponentam celične površine) dosežemo večjo specifičnost takšnega načina vnosa (20).

V primerjavi z virusnimi vektorji so nevirusni načini vnosa varnejši, vendar manj specifični in učinkoviti. K povečanju specifičnosti nevirusnega vnosa pripomorejo fizikalne metode, kot so ultrazvok, biolistika, magnetofekcija, terapevtska hipertermija in elektroporacija.

Fizikalne metode vnosa genskega materiala v ciljno celico:

a) Vnos genov z ultrazvokom: z ultrazvočnimi valovi dosežemo začasno prepustnost celične membrane za makromolekule. Metoda je varna in ponovljiva, vendar so potrebne nadaljnje študije za njeno izboljšanje (25).

b) Vnos genov z biolistiko: s pomočjo specifične pištole vnesemo v celice delce iz težke kovine, obdane s plazmidno DNA. Metoda ni toksična in je relativno učinkovita, vendar so potrebne nadaljnje študije takšnega načina vnosa genov v tkiva (26, 27).

c) Vnos genov z magnetofekcijo: na magnetne nanodelce reverzibilno vežemo DNA in jih dovedemo do ciljne celice s pomočjo magnetnega polja. Takšen način vnosa omogoča zelo hitro in učinkovito transfekcijo genov. Samo magnetno polje pa ne omogoča prenosa gena skozi celično membrano, temveč le močno akumulacijo magnetnih nanodelcev z vezanim genom na površini membrane (28).

d) Vnos genov s hipertermijo: s kontroliranim povečevanjem temperature na ciljnem tumorskem tkivu omogočimo tako toksični efekt zaradi povišanja temperature kot povečano prepustnost membrane celice za makromolekule. Hipertermija naj bi povzročila izgubo medsebojnih kontaktov endotelnih celic tumorja. To naj bi privedlo do širjenja medceličnih prostorov v tumorju in tako omogočilo vnos protitumorskih učinkovin v globino tumorja (28).

e) Vnos genov z elektroporacijo: z dovajanjem zunanjega električnega polja se poveča prepustnost celične membrane za majhne in večje molekule, ki drugače ne prehajajo prosto skozi membrano. Ko zunanje električno polje izklopimo, se membrana celice ponovno vrne v prvotno stanje in metabolno aktivna celica izraža tako vneseni gen. Elektroporacija predstavlja najbolj obetavno alternativo virusni transfekciji na področju genske terapije, saj lahko tako učinkovito

vnašamo prosto DNA brez prisotnih kemičnih dodatkov. Metoda je preprosta, izbira primernih parametrov električnih pulzov pa nam omogoča varen vnos genov v ciljno celico (29).

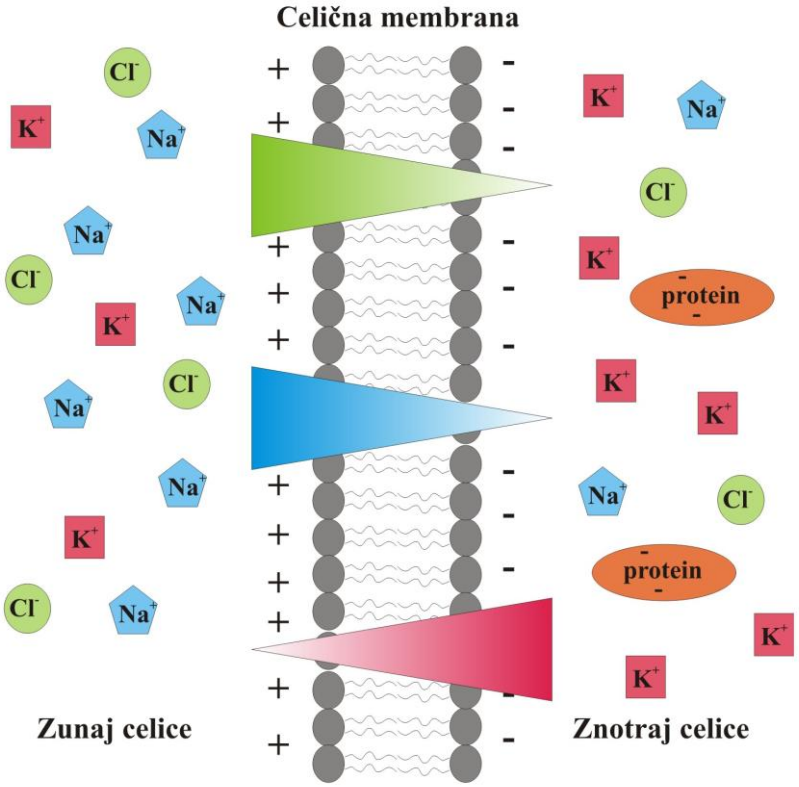
3 ELEKTROPORACIJA

Elektroporacija je metoda, ki z uporabo visokonapetostnih električnih pulzov omogoča vnos molekul v celico. Že leta 1972 so bile prvič opisane permeabilne spremembe na lipidnih veziklih, ki so nastale zaradi dovajanja zunanjega električnega polja (30). Kasneje so pokazali, da pride ob izpostavitvi celice dovolj visoki jakosti zunanjega električnega polja do začasnih strukturnih sprememb celične membrane, ki zato postane prepustna za ione, molekule in makromolekule, ki drugače ne prehajajo skoznjo (30 - 32). Pravimo, da je membrana permeabilizirana, zato se za metodo uporablja tudi izraz elektropermeabilizacija. Uspešnost elektroporacije lahko določamo z vnosom majhnih molekul, ki sicer ne prehajajo skozi nepermabilizirano membrano. Med najpogosteje uporabljenimi molekulami je fluorescentna molekula propidijevega jodida (PI) (33 - 42).

3.1 Transmembranska napetost

3.1.1 Mirovalna transmembranska napetost

Poglavitno vlogo pri vzpostavitvi transmembranske napetosti ima Na^+/K^+ črpalka v celični membrani, ki z aktivnim transportom prečrpa tri ione Na^+ iz celice in dva iona K^+ v celico, pri čemer pride do naraščanja koncentracije Na^+ ionov zunaj celice in koncentracije K^+ ionov znotraj celice (Slika 3). S prehodom K^+ ionov v smeri svoje nižje koncentracije po trajno prepustnem K^+ kanalu v membrani se sčasoma vzpostavi ravnotežno stanje, ki mu pravimo mirovalna transmembranska napetost, katere vrednost je med –40 in –90 mV (43).



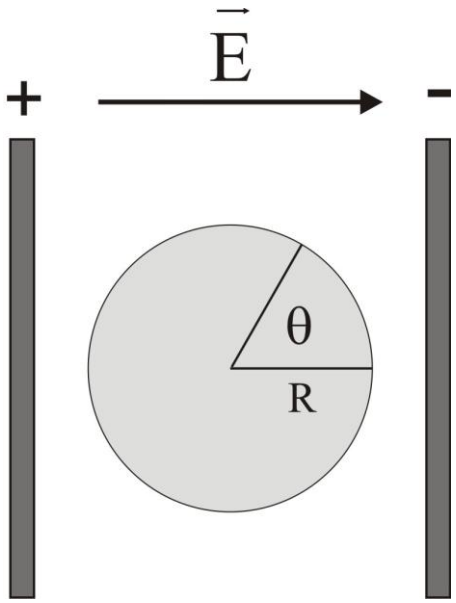
Slika 3: Mirovalna transmembranska napetost na celični membrani. Zaradi majhnega primanjkljaja pozitivnih ionov v citoplazmi je notranji del celične membrane rahlo negativno nabit v primerjavi z njenim zunanjim delom.

3.1.2 Vsiljena transmembranska napetost

Vsiljena transmembranska napetost nastane zaradi prerazporeditve ionov v električnemu polju. Ob izpostavitvi celice električnemu polju nastanejo na njeni membrani spremembe, ki so posledica vsiljene transmembranske napetosti (44 - 46). Te spremembe omogočijo vnos molekul, ki drugače ne bi prehajale skozi membrano, v celico. Vrednost, ki jo vsiljena transmembranska napetost doseže na membrani okrogle celice v homogenem električnem polju, lahko opišemo s Schwanovo enačbo (47):

$$U_{vsiljena} = \frac{3}{2} ER\cos\theta \qquad (1)$$

kjer je *E* jakost zunanjega električnega polja, *R* polmer celice in *cos θ* kosinus kota med smerjo električnega polja ter daljico, ki povezuje središče celice in obravnavano točko na membrani (Slika 4).



Slika 4: Prikaz parametrov celice, izpostavljene električnemu polju.

Schwanova enačba nam omogoča razumevanje vpliva posameznih parametrov na membrano celice. S povečevanjem jakosti električnega polja pride do sorazmerno linearnega povečanja vsiljene transmembranske napetosti. Večje celice bodo pri enaki jakosti električnega polja izpostavljene večji transmembranski vsiljeni napetosti (48). Če celica ni okrogla, velja Schwanova enačba le približno (49 - 51).

Pri dovolj visoki vsiljeni transmembranski napetosti se prepustnost in prevodnost celične membrane močno povečata. Pravimo, da je bila dosežena pragovna vrednost transmembranske napetosti, katere vrednosti se nahajajo med 200 mV in 1 V (44, 46, 52 - 55).

3.2 Mehanizmi elektroporacije

Znanih je več teoretičnih mehanizmov, ki skušajo razložiti povečano prepustnost membrane kot posledico izpostavitve celice električnemu polju. Nekatere teorije so trdile, da naj bi zaradi vpliva električnega polja prišlo do denaturacije membranskih

kanalov, tvorbe razpok med mikropodročji v fosfolipidnem dvosloju ali do elektrokompresije membrane, vendar nobene izmed teh teorij še niso potrdili z eksperimentalnimi študijami (56). Kljub temu, da na celični membrani neposredno opazovanje por ni mogoče, je danes najbolj uveljavljen model elektroporacije formacija hidrofilnih por (57, 58), kar so skušali pokazati s simulacijo molekularne dinamike fosfolipidnih dvoslojev v prisotnosti električnega polja (59). Kljub temu, da celične membrane ne sestavlja le fosfolipidni dvosloj, ampak je membrana kompleksnejša, vsebuje številne proteine in je povezana s citoskeletom (2), so pokazali, da se enake strukturne spremembe dogajajo tudi na membrani, vendar le v fosfolipidnem delu (44, 56, 60).

Elektroporacijo celične membrane lahko opišemo kot reverzibilno in ireverzibilno. Izpostavitev celice zunanjemu električnemu polju nad pragovno vrednostjo vsiljene transmembranske napetosti privede do povečanja prevodnosti celične membrane in pojava sprememb na membrani, ki trajajo, dokler je prisotno zunanje električno polje. Nekaj milisekund po tem, ko zunanje električno polje pade pod pragovno vrednost vsiljene transmembranske napetosti, se membrana začne vračati v prvotno stanje, čemur sledi okrevanje. Pojav opišemo kot reverzibilno elektroporacijo (48). Če pa je zunanje električno polje prisotno dalj časa ali če je vrednost vsiljene transmembranske napetosti visoko nad pragovno vrednostjo, potem po prenehanju dovajanja električnega polja membrana celice ne okreva, kar vodi v celično smrt. V tem primeru govorimo o ireverzibilni elektroporaciji (61).

3.3 Vpliv dejavnikov na elektroporacijo

Na uspešnost elektroporacije vplivajo številni dejavniki, kot so parametri električnih pulzov, lastnosti elektroporacijskega medija, lastnosti celic idr. S številnimi študijami so skušali ugotoviti vpliv različnih dejavnikov, ki bi lahko pripomogli k izboljšanju in razumevanju metode.

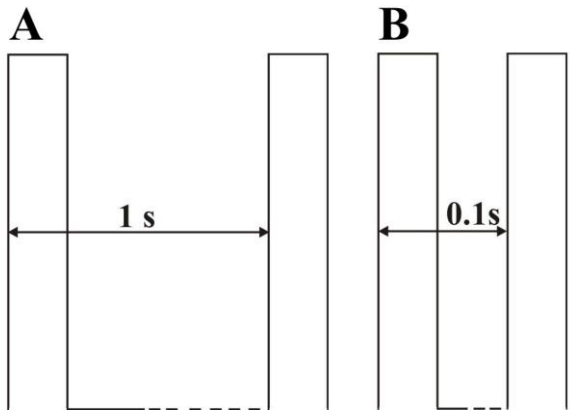
3.3.1 Vpliv električnih pulzov na elektroporacijo

Za uspešen vnos molekul v celico s pomočjo elektroporacije sta potrebna primerna izbira parametrov električnih pulzov, da dosežemo uspešnost metode, in čim večje preživetje celic. Zato morajo biti parametri izbrani tako, da je dosežena enaka ali višja pragovna vrednost vsiljene transmembranske napetosti, ki pa hkrati ne sme preseči vrednosti, zaradi katere bi prišlo do trajne poškodbe celične membrane.

Delež celic, v katere uspešno vnašamo molekule z elektroporacijo, je odvisen od izbranih parametrov električnih pulzov, kot so število električnih pulzov (33, 48, 62 - 64), trajanje posameznega pulza (48, 63, 64), jakost električnega polja (48, 63, 64) in izbrana ponavljalna frekvenca (65).

S povečevanjem števila pulzov, podaljšanjem trajanja električnega pulza in s povečevanjem jakosti električnega polja narašča tudi delež uspešno elektroporiranih celic.

S spreminjanjem ponavljalne frekvence pa spreminjamo hitrost dovajanja električnega pulza (Slika 5).

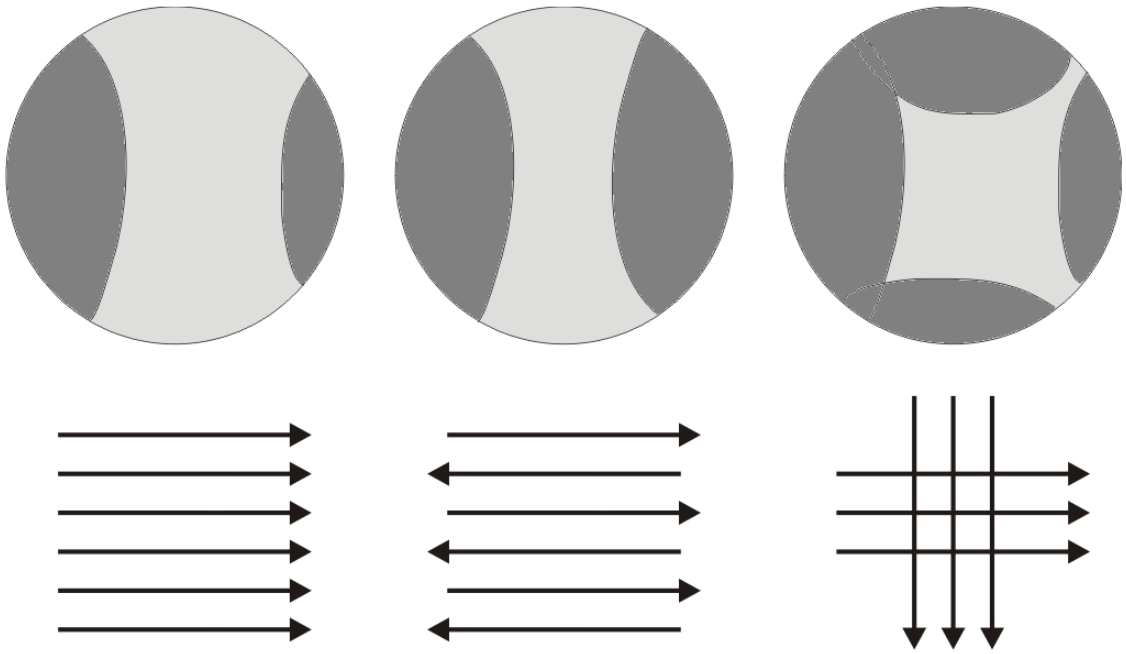


Slika 5: Shematski prikaz dveh različnih ponavljalnih frekvenc. (A) 1 Hz; (B) 10 Hz.

Če povečujemo ponavljalno frekvenco ter hkrati povečujemo število in zmanjšujemo trajanje pulzov, tako da ostaja čas med začetkom prvega in koncem drugega pulza enak, potem se delež permeabiliziranih celic zmanjšuje (62). S povečevanjem ponavljalne

frekvence pri enakem trajanju in številu pulzov pa ne vplivamo bistveno na delež uspešno permeabiliziranih celic (66). Pri večjih frekvencah je potrebna višja napetost za dosego maksimalnega deleža permeabiliziranih celic, vsaka frekvenca ima pri drugačni napetosti namreč drugačno vrednost maksimalnega vnosa majhnih molekul (65).

Prav tako je pomembna smer dovajanja električnih pulzov. Z dovolj zmogljivim generatorjem električnih pulzov lahko dovajamo električne pulze v različnih smereh in tako omogočimo elektropermeabilizacijo membrane na različnih predelih (Slika 6), s čimer izboljšamo uspešnost vnosa molekul (50, 67 - 69).



Slika 6: Shematski prikaz celice, izpostavljene različnim smerem dovajanja električnih pulzov. Površina membrane, na kateri membranski potencial presega kritični transmembranski potencial, je označena temneje. Smer električnega polja je prikazana s puščicami.

S takšnim načinom povečamo površino elektropermeabilizirane membrane, saj je celična membrana uspešno elektropermeabilizirana le na delih, ki ležijo pravokotno na smer dovajanja električnega polja (glej Enačbo 1 in Sliko 6).

Vrednosti izbranih parametrov električnih pulzov, s katerimi dosežemo uspešno elektroporacijo, pa so odvisne od orientacije celice v električnem polju (50), velikosti in oblike celice (48) ter gostote celic (70 - 72).

3.3.2 Vpliv drugih dejavnikov na elektroporacijo

Poleg parametrov električnih pulzov na elektropermeabilizacijo celic lahko vplivajo tudi številni drugi dejavniki. Mednje štejemo vpliv temperature (73), vpliv oksidativnega stresa (74), vpliv osmotskega pritiska (34, 75), vpliv celičnega cikla (35) in vpliv prevodnosti elektroporacijskega medija (76).

3.4 Področja uporabe elektroporacije

Permeabilne spremembe celične membrane, ki nastanejo zaradi dovajanja zunanjega električnega polja, omogočajo različne aplikacije:

a) Elektrofuzija: je metoda zlivanja celic z dovajanjem električnih pulzov, s katero lahko pripravljamo monoklonska protitelesa (77). Za uspešno elektrofuzijo je potrebna vzpostavitev fuzogenega stanja celične membrane in zagotovitev stika med celicama (78, 79).

b) Sterilizacija hrane: z elektroporacijo lahko brez toplotne obdelave inaktiviramo morebitno prisotne mikroorganizme v tekočih prehrambenih pripravkih (80, 81).

c) Ireverzibilna elektroporacija: z dovajanjem električnih pulzov lahko povzročimo destabilizacijo transmembranske napetosti v takšni meri, da nastanejo v celični membrani okvare, ki vodijo v celično smrt (82). Z ireverzibilno elektroporacijo tako lahko brez toplotnega segrevanja selektivno uničimo določeno tkivo (83, 84).

d) Elektrokemoterapija: je metoda, s katero zdravimo kožne, podkožne in tudi globje ležeče tumorje (85, 86). Temelji na lokalnem ali sistemskem vnosu kemoterapevtika, ki mu sledi dovajanje električnih pulzov na mestu obolenja. Tako omogočimo vnos kemoterapevtika, ki drugače ne prehaja ali slabo prehaja celično membrano, in s tem dosežemo večjo citotoksičnost zdravil, kot sta bleomicin in cisplatin (87).

e) Genska elektrotransfekcija: z dovajanjem električnih pulzov lahko v celice vnesemo tudi večje molekule, kot je na primer DNA (31, 88, 89). Metoda se uporablja v genski terapiji (90) ali pri imunizaciji z golo DNA (91, 92).

4 GENSKA ELEKTROTRANSFEKCIJA

Začetki genske elektrotransfekcije segajo v zgodnja osemdeseta leta, ko je bil prvič opisan primer vnosa DNA v celice *in vitro* (31, 93). Od takrat so raziskave precej napredovale in danes je genska elektrotransfekcija pogosto uporabljana metoda v predkliničnem in tudi kliničnem okolju.

4.1 Predklinične in klinične študije genske elektrotransfekcije

Prednost genske elektrotransfekcije je predvsem preprost in varen način vnosa genskega materiala *in vivo*. Kljub temu, da nam številne biokemične metode prav tako omogočajo vnos genov v celice, so namreč mnoge med njimi manj učinkovite, zapletene ali pa imajo neželene stranske učinke, ki so posledica vnosa kemičnih ali virusnih dodatkov (19, 94). Za vnos genov *in vivo* se metoda uporablja že od leta 1991 (95) in danes je v teku velika kopica predkliničnih študij genske elektrotransfekcije.

4.1.1 Predklinične študije

Predklinične študije *in vivo* so raziskave na živalskih modelih, s katerimi preverjamo učinkovitost in toksičnost neke metode ali zdravila. Boljše razumevanje mehanizmov genske elektrotransfekcije na živalskih modelih je tako pripomoglo k hitrejšemu prehodu k do zdaj maloštevilnim kliničnim študijam. Z dovajanjem električnih pulzov so uspešno vnesli DNA v različna tkiva, kot so mišice (96 - 100), tumorji (29, 101 - 105), jetra (106), koža (107, 108), roženica (109, 110), srčno tkivo (111), žile (112) in pljuča (113 - 116). S terapevtskega vidika in vidika dostopnosti so najprimernejše ciljno tkivo za gensko elektrotransfekcijo mišice in tumorji (117 - 119).

Mišice so pomembno ciljno tkivo zaradi lahke dostopnosti, dobre prekrvavljenosti in dolgotrajnega učinka izražanja vnesenega gena. Mišično tkivo je s svojo sekretorno funkcijo postalo pomembno ciljno tkivo za vnos terapevtskih genov z dovajanjem električnih pulzov, katerih produkti delujejo lokalno in sistemsko (118).

Nasprotno pa je za vnos terapevtskih genov z dovajanjem električnih pulzov v tumorsko tkivo značilno nizko in kratkotrajno izražanje terapevtskega gena, kar je verjetno posledica hitro delečih se tumorskih celic (29, 38, 101).

4.1.2 Klinične študije

Prva klinična študija faze I vnosa terapevtskega gena z gensko elektrotransfekcijo je potekala pri pacientih z metastatsko obliko melanoma. Z vnosom plazmida, ki kodira gen za IL-12 (pIL-12) z električnimi pulzi, so pri pacientih spremljali varnost, toleranco in učinek terapije. Po injiciranju plazmidne DNA v tumor je sledilo dovajanje šestih pulzov trajanja 100 μs, z jakostjo električnega polja 1300 V/cm, in sicer 3-krat dnevno, 8 dni zapored. Pacienti so ob tem poročali le o blagi prehodni bolečini oziroma neudobju. Zaključki raziskave so bili, da je genska elektrotransfekcija s pIL-12 varna in dobro tolerirana ter da je opaziti delno ali popolno regresijo oddaljenih in netretiranih

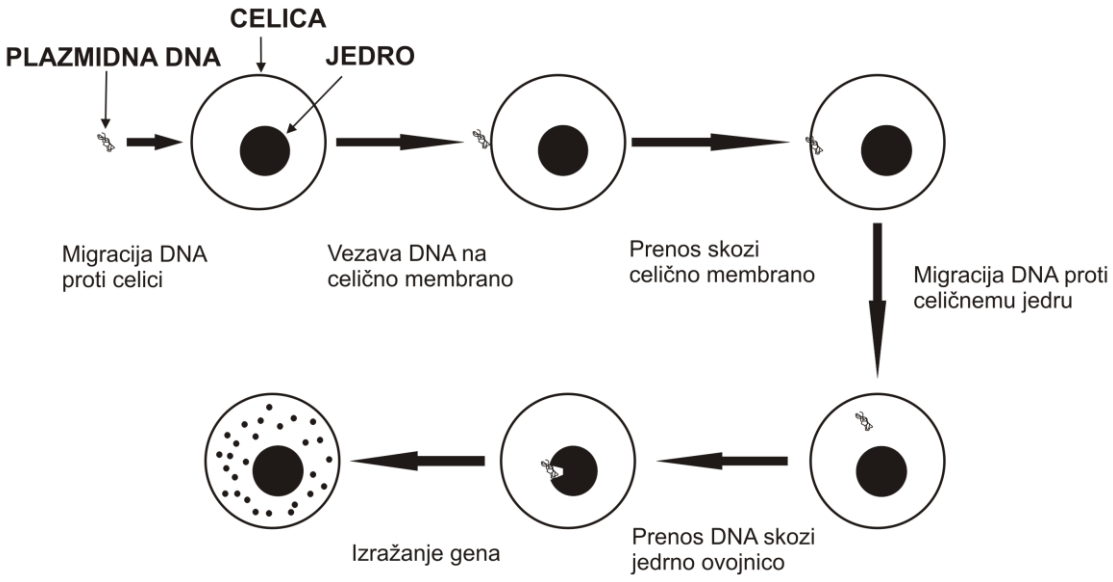
melanomov, kar kaže na sistemski učinek terapije. Na podlagi rezultatov že načrtujejo prehod k študiji klinične faze II (39).

Druga klinična študija faze I je prav tako potekala pri pacientih z metastatsko obliko melanoma (http://clinicaltrials.gov). Z dovajanjem električnih pulzov so vnašali plazmid, ki kodira gen za IL-2 (pIL-2). Rezultati študije še niso objavljeni.

Trenutno se izvaja več kliničnih študij genske elektrotransfekcije (http://clinicaltrials.gov), kjer spremljajo varnost, toleranco in učinkovitost imunizacije z DNA cepivom, ki ga vnašajo z električnimi pulzi.

4.2 Mehanizmi genske elektrotransfekcije

Natančni mehanizmi vnosa DNA v celico s pomočjo električnih pulzov še vedno niso popolnoma pojasnjeni. Znano je, da je ključni dejavnik uspešno permeabilizirana celična membrana, na katero se veže DNA, čemur sledi prenos DNA skozi permeabilizirano membrano (katere mehanizem še ni jasen), migracija DNA po citoplazmi celice do celičnega jedra, prenos skozi jedrno ovojnico in nato izražanje gena (Slika 7) (36).



Slika 7: Shematski prikaz posameznih korakov genske elektrotransfekcije (120).

Proces vnosa DNA v celico z dovajanjem električnih pulzov so študirali na nivoju umetno formiranih fosfolipidnih dvoslojev, velikih enoslojnih veziklov, celic *in vitro* ter na različnih tkivih *in vivo*.

Po najpreprostejšem predlaganem mehanizmu naj bi se DNA prenesla v celico z elektroforetsko silo skozi pore na membrani, ki naj bi nastale kot posledica dovajanja električnih pulzov (121, 122). Kljub temu, da so pore opazne na veziklih, ki predstavljajo strukturo, podobno celični membrani, na živih celicah por niso uspeli pokazati, zato ta model še vedno ostaja nerazjasnjen (123).

Po drugem predlaganem mehanizmu naj bi DNA tvorila začasen kompleks s fosfolipidi v membrani, in sicer ob robu hidrofilnih por, nastalih zaradi dovajanja električnih pulzov (124). Z raziskavo na nivoju ene same celice so pokazali, da se negativno nabita DNA molekula ob dovajanju električnih pulzov zaradi elektroforetske sile nakopiči predvsem na strani membrane, ki gleda proti katodi, tvori stabilen kompleks z membrano in se prenese v citoplazmo celice (125).

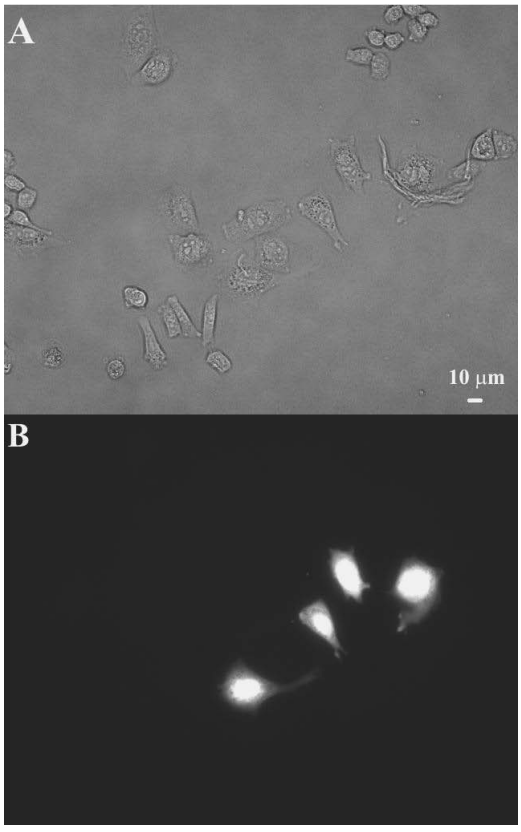
Nenazadnje so tudi skušali dokazati, da DNA po dovajanju električnih pulzov vstopa v celico s procesom endocitoze. Velike enoslojne vezikle so izpostavili dovolj visoki jakosti električnega polja in pokazali, da DNA ni vstopila v vezikel skozi poro, temveč v endosomu podobni strukturi (126). Danes skušajo potrditi predlagani mehanizem s študijami *in vitro* na celicah (127).

Pokazali so tudi povečan preskok fosfolipidov iz enega sloja celične membrane v drugega (Slika 2), ki je nastalo zaradi dovajanja električnih pulzov. Tako gibanje naj bi povzročilo večjo prepustnost membrane in s tem pripomoglo k boljšemu vnosu molekule DNA v celico (128).

Poleg prenosa DNA skozi celično membrano je za uspešno transfekcijo pomembna tudi stabilnost DNA v citoplazmi celice, saj jo lahko razgradijo znotrajcelične DNaze (129). Pri prenosu DNA do jedra sodeluje celični citoskelet (130), pokazali pa so, da uspešnost genske elektrotransfekcije lahko povečamo z dodajanjem lokalizacijskega jedrnega signalnega peptida na DNA, ki jo želimo prenesti v jedro celice (131).

Z dovajanjem električnih pulzov lahko v celice vnašamo reporterske gene (kot je gen za zeleno fluorescirajoči protein GFP -Slika 8, luciferazo, β-galaktozidazo,

kloramfenikol acetil transferazo), terapevtske gene (kot je gen za interlevkine, INF-α, timidin kinazo, p53 itd.) ali fluorescentno označeno DNA. Reporterski geni omogočajo preučevanje mehanizmov genske elektrotransfekcije in določanje uspešnosti metode, medtem ko z vnosom terapevtskih genov (gen za interlevkine, INF-α, timidin kinazo, p53 itd.) sežemo korak naprej, saj gledamo učinek delovanja terapevtskega gena (npr. zaustavitev rasti tumorja ali popolna regresija tumorja) (117, 118, 132 - 134). S fluorescentno označeno DNA pa lahko opazujemo vezavo DNA na celično membrano po dovajanju električnih pulzov ali njen prenos po citoplazmi do jedra (36, 66, 125, 135).



Slika 8: Prikaz genske elektrotransfekcije. Z dovajanjem serije 8 električnih pulzov, trajanja 1 ms, s ponavljalno frekvenco 1 Hz smo v celice vnesli plazmidno DNA, ki nosi zapis za GFP. (A) Fazno kontrastna slika tretiranih celic 24 h po dovajanju električnih pulzov in (B) fluorescentna slika istih tretiranih celic, v katerih se prepisuje GFP protein (belo). Celice smo opazovali z invertnim fluorescentnim mikroskopom pri 200-kratni povečavi. Merilo = 20 μm.

4.3 Dejavniki, ki vplivajo na gensko elektrotransfekcijo

Na učinkovitost genske elektrotransfekcije vpliva vrsta dejavnikov, med drugimi: parametri električnih pulzov (62, 68, 136, 137), temperatura (138), sestava elektroporacijskega medija (139 - 143) ter tip in biološko stanje celic (35, 50). V bolj kompleksnih celičnih sistemih, kot so tkiva in organi, pa na učinkovitost genske elektrotransfekcije vpliva tudi struktura tkiva, interakcija celic s komponentami zunajceličnega ogrodja (proteoglikani, polisaharidi, kolagenska in druga vlakna) (144) in predvsem zmanjšana mobilnost DNA v zunajceličnem ogrodju (119, 145, 146). Slaba difuzija DNA v tkivu onemogoča tvorbo kompleksa DNA z membrano celice, ki je nujni pogoj za uspešno gensko elektrotransfekcijo.

4.3.1 Vpliv električnih pulzov na gensko elektrotransfekcijo

Pomemben vpliv na gensko elektrotransfekcijo ima primerna izbira parametrov električnih pulzov, kot so število pulzov, trajanje pulza, jakost električnega polja in ponavljalna frekvenca (64, 147).

Z električnimi pulzi, ki trajajo nekaj milisekund (ms) (62, 148), ali s kratkimi mikrosekundnimi (µs) pulzi ob povečanju jakosti električnega polja dosežemo uspešno gensko elektrotransfekcijo in zadovoljivo preživetje celic (31). Eden izmed dejavnikov za učinkovito gensko elektrotransfekcijo *in vitro* in *in vivo* je torej trajanje električnega pulza (31, 106). Ker je DNA polianionska molekula, nanjo v električnem polju deluje elektroforetska sila (149). S študijo vpliva trajanja električnih pulzov na gibanje DNA v agaroznem gelu so pokazali, da se elektromobilnost DNA povečuje z daljšanjem pulza in večanjem amplitude pulza. Torej dalj trajajoči in močnejši pulzi pospešijo hitrost gibanja DNA v električnem polju (146), kar je še posebej pomembno pri vnosu DNA *in vivo*, kjer gosta struktura celičnega okolja vpliva na zmanjšano elektromobilnost DNA (145).

S številnimi študijami *in vivo* so pokazali pomemben vpliv uporabe kombinacije visokonapetostnega (HV) in nizkonapetostnega (LV) električnega pulza na uspešnost

genske elektrotransfekcije (107, 137, 150 - 152). Prvi pulz (HV, kratkega trajanja) omogoči elektropermeabilizacijo membrane, medtem ko drugi pulz (LV, daljšega trajanja) membrane ne permeabilizira, temveč poveča elektroforetsko mobilnost DNA (149, 153) in vpliva tudi na preživetje celic (154). Za uspešno gensko elektrotransfekcijo je torej pomembna primerna izbira kombinacije HV in LV pulza. S povečevanjem amplitude HV pulza in podaljševanjem trajanja in amplitude LV pulza uspešnost genske elektrotransfekcije *in vivo* narašča (151). Kljub temu, da so nekateri avtorji postavili pod vprašaj pomembnost elektroforetske mobilnosti pri genski elektrotransfekciji *in vivo* (155), študije *in vitro* poudarjajo pomemben vpliv elektroforetske mobilnosti pri manjši koncentraciji DNA, saj je v okolju *in vivo* količina DNA v bližini celice majhna (136, 156, 157).

Pomemben dejavnik, ki vpliva na uspešnost genske elektrotransfekcije je tudi spreminjanje smeri dovajanja električnih pulzov. V prisotnosti zunanjega električnega polja se negativno nabita DNA nakopiči le na tistem delu membrane celice, ki se nahaja na strani katode (125). Z obračanjem smeri dovajanja električnih pulzov pa povečamo površino elektropermeabilizirane membrane (glej Sliko 6) in uspešnost genske elektrotransfekcije močno poraste (66, 68, 69).

4.3.2 Vpliv ionov na gensko elektrotransfekcijo

Divalentni kationi (kot so kalcijevi Ca^{2+} in magnezijevi ioni Mg^{2+}) naj bi pripomogli k zmanjšanju elektrostatske odbojne sile med negativno nabito DNA in negativno nabito celično membrano pri dovajanju električnih pulzov s čimer naj bi povečali uspešnost genske elektrotransfekcije (139, 140, 158). Moč vezave DNA na negativno nabiti substrat ob prisotnosti različnih divalentnih kationov je odvisna od premera divalentnega kationa. Pokazali so, da Mg^{2+} ali Ca^{2+} ne vežejo ali slabo vežejo DNA na hidroksiliran in negativno nabiti substrat SiO_X. Avtorji razlagajo, da ti kationi zaradi svoje velikosti ne dosežejo hidroksilnih skupin, ki se nahajajo na substratu, in posledično ne omogočajo boljše vezave DNA. Kljub vsemu poudarjajo, da je mehanizem vezave DNA na negativno nabiti substrat bolj kompleksen (159, 160). S

študijo na anionskih veziklih, katerih fosfolipidni dvosloj je podoben membrani celice, so pokazali, da se ob povečevanju Ca^{2+} ionov v elektroporacijskem mediju količina vezane DNA na membrani vezikla poveča (161, 162).

Vpliv različnih ionov na uspešnost genske elektrotransfekcije opisujejo številne študije *in vitro* ter *in vivo*, vendar rezultati teh raziskav niso konsistentni (31, 139 - 143, 158).

Vpliv Mg^{2+} na vezavo DNA z membrano celice pri dovajanju električnih pulzov je bil opisan že v začetku osemdesetih let (93). Z radioaktivno označeno DNA so pokazali, da se s povečanjem koncentracije Mg^{2+} (do 20 mM) v elektroporacijskem mediju povečuje količina vezane DNA na membrani celice (163).

Kljub večji količini DNA na membrani pa uspešnost genske elektrotransfekcije pada z naraščanjem koncentracije Mg^{2+} (31, 153). Pri 30 mM in višji koncentraciji Mg^{2+} je delež uspešno transfeciranih celic po dovajanju električnih pulzov močno znižan (31, 143). Toda ob povečevanju Mg^{2+} od 1 do 8 mM koncentracije je druga raziskovalna skupina opazila povečan delež uspešno transfeciranih celic *in vitro* (139). Prav tako so opazili povečan delež uspešno transfeciranih celic pri povečevanju Ca^{2+} na celicah kože *in vivo* (158). S kasnejšimi študijami na mišičnih celicah *in vivo* so pokazali, da s povečevanjem Ca^{2+} ionov (142) ali Na^{+} ionov (141) uspešnost genske elektrotransfekcije upada. Avtorji razlagajo, da zaradi povečane vezave pozitivno nabitih ionov na molekulo DNA njena elektroforetska mobilnost v električnem polju upade (141).

Za uspešno gensko elektrotransfekcijo je poleg vezave DNA na celično membrano pomemben tudi njen uspešen prenos po citoplazmi celice do jedra. Mg^{2+} ioni so pomembni aktivatorji znotrajceličnih DNaz, encimov, ki razgradijo DNA molekulo (96, 129). Z inhibicijo slednjih so povečali učinkovitost genske elektrotransfekcije (164).

4.3.3 Vpliv temperature na gensko elektrotransfekcijo

Temperatura ima pomemben vpliv na fluidnost celične membrane (2) in s tem na uspešnost elektropermeabilizacije membrane ter posledično na gensko

elektrotransfekcijo. Pri višji temperaturi (37ºC) so pokazali, da je membrana celice bolj fluidna in posledično elektropermeabilizirana pri nižji jakosti električnega polja, kot pri nižji temperaturi (4ºC) (73). Vpliv temperature na gensko elektrotransfekcijo pa variira v odvisnosti od vrste celice, saj ima membrana različne domene in različno vsebnosti holesterola (2). Tako se lahko uspešnost genske elektrotransfekcije pri določeni temperaturi zelo razlikuje pri različnih celičnih linijah.

Na celicah ovarija kitajskega hrčka (CHO) so pokazali, da, ko celice pred dovajanjem električnih pulzov izpostavimo nižji temperaturi (4ºC), pridobimo večji delež uspešno transfeciranih celic. Pomembno vlogo ima tudi temperatura po elektroporaciji, saj z višanjem le-te (do 37ºC) omogočimo boljše okrevanje celične membrane in s tem boljše preživetje celic (138). Študije z drugo celično linijo pa so pokazale bolj uspešno gensko elektrotransfekcijo, če so celice pred dovajanjem električnih pulzov inkubirane na 20ºC, in sicer je bila uspešnost genske elektrotransfekcije od 1.6−50 krat višja v primerjavi s celicami, inkubiranimi na 0ºC (165).

4.3.4 Vpliv lastnosti elektroporacijskega medija na gensko elektrotransfekcijo

Na izražanje DNA močno vpliva osmolarnost medija tako med kot po dovajanju električnih pulzov. S študijo genske elektrotransfekcije v medijih različne osmolarnosti so pokazali, da je delež uspešno transfeciranih celic v hipoosmoznem mediju dvakrat višji in v hiperosmoznem mediju dvakrat nižji kot v isoosmolarnem mediju (75). Zaradi nabrekanja celice v hipoosmoznem okolju naj bi nastal hidrodinamičen pretok vode, ki naj bi povečal vnos DNA v celico ob dovajanju električnih pulzov.

4.3.5 Vpliv lastnosti celic na gensko elektrotransfekcijo

Jedrna ovojnica predstavlja kritično bariero za gensko transfekcijo. Pri večini nevirusnih načinov vnosa DNA v celico ravno prehod DNA iz citoplazme v jedro predstavlja temeljno omejitev za uspešno gensko transfekcijo (166). Pokazali so, da je genska

elektrotransfekcija najbolj uspešna, če se celica nahaja v fazi G2/M celičnega cikla (35). V G2 fazi celičnega cikla poteka aktivna biosinteza mikrotubulov, ki so potrebni v procesu mitoze (2). Zato se celični volumen poveča in večje celice so pri enaki jakosti električnega polja izpostavljene večji transmembranski vsiljeni napetosti (48). Poleg tega se v fazi M (mitoza) jedrna ovojnica razgradi in z elektroporacijo vnesena DNA lažje doseže jedro celice.

Poleg tipa in oblike celice ter faze celičnega cikla je za gensko elektrotransfekcijo pomembna tudi gostota celic. Tesen kontakt med celicami fizično onemogoča stik DNA s celico, zato je uspešnost genske elektrotransfekcije gostih celičnih kultur manjša (37).

Pomembno vlogo pri prenosu DNA čez elektropermeabilizirano celično membrano celic *in vitro* ima prav tako adenozin difosfat (ADP), medtem ko adenozin trifosfat (ATP) vpliva predvsem na prenos DNA po citoplazmi do jedra in na ekspresijo. Genska elektrotransfekcija je torej proces, ki ni odvisen samo od parametrov električnih pulzov, temveč tudi od stanja in lastnosti celice (167).

4.3.6 Vpliv drugih dejavnikov na gensko elektrotransfekcijo

Pri virusnem načinu vnosa genov velikost DNA ni pomembna, medtem ko pri genski elektrotransfekciji njena učinkovitost pada z naraščanjem velikosti DNA. Pomemben dejavnik je prav tako koncentracija DNA in čas njene aplikacije. Z večanjem koncentracije DNA linearno narašča tudi delež uspešno transfeciranih celic (64, 153, 165), vendar do določene meje, ko pa postane prevelika količina DNA toksična za celice (64, 147). Kopičenje DNA ob celični membrani in njen prenos skozi membrano je mogoč le, če je DNA prisotna med dovajanjem električnih pulzov (64, 153). Topologija DNA nima vpliva na prenos skozi membrano, vendar pa ima vpliv na njeno stabilnost (139).

Z dodajanjem telečjega seruma celicam takoj po dovajanju električnih pulzov lahko izboljšamo preživetje in delež uspešno transfeciranih celic (168).

Pri vnosu DNA v tkivo z gensko elektrotransfekcijo na uspešnost vpliva koncentracija DNA v ciljnem tkivu. Z injiciranjem DNA v tkivo dosežemo maksimalno

količino DNA na mestu dovajanja električnih pulzov in omogočimo visoko kopičenje DNA na membrani celic (96). Prav tako z dodajanjem encimov, ki razgradijo komponente zunajceličnega ogrodja, omogočimo boljšo mobilnost DNA skozi tkivo do celic. Pokazali so, da se z injiciranjem encima hialuronidaze v mišično tkivo 2 h pred dovajanjem električnih pulzov genska elektrotransfekcija izboljša za 40 % (97). Ker je za uspešnost genske elektrotransfekcije *in vivo* pomembna tudi primerna razporeditev električnega polja v ciljnem tkivu, je nujna primerna izbira elektrod. Danes poznamo več tipov elektrod, med katerimi se najpogosteje uporabljajo igelne elektrode, s katerimi dosežemo globlje ležeča tkiva, ter paralelne ploščate elektrode za dovajanje električnih pulzov na površini kože (169).

4.4 Biotehnološke aplikacije genske elektrotransfekcije *in vitro*

Velike količine specifičnega proteina za biotehnološke ali medicinske namene najlažje pridobimo s transformacijo bakterij. Številne bakterije so uspešno transformirali z gensko elektrotransfekcijo (170 - 175), prav tako tudi gram pozitivne endofitske bakterije (kot je *Bacillus mojavensis*), ki se sicer težje transformirajo (176). Ker je genska elektrotransfekcija preprosta, učinkovita in hitra metoda, bi lahko kaj kmalu popolnoma zamenjala ostale metode transformacij bakterij (177).

5 TRIDIMENZIONALNI CELIČNI MODELI

Gojenje celic v celični kulturi je proces, kjer prokariontskim ali evkariontskim celicam omogočamo rast v nadzorovanih pogojih. Sam proces omogoča, da se posamezna celica obnaša kot zaključena enota in da je sposobna delitve in rasti, dokler ji to zanjo ugodni pogoji omogočajo. Celične kulture se že štiri desetletja rutinsko uporabljajo v številnih laboratorijih po svetu in danes poznamo več kot sto različnih celičnih linij, s pomočjo katerih se odvijajo različne raziskave na področjih, kot so virologija, bakteriologija, onkologija, genetika in mnoga druga (178).

Raziskovalci so se med delom s celičnimi kulturami soočili s problemom, da celice v klasični kulturi nimajo biokemičnih in morfoloških lastnosti celic tkiva. Te celice imajo ohranjene predvsem osnovne lastnosti, ki celici omogočajo preživetje v kulturi. Zato se je pojavila ideja o drugačnem gojenju celic, v tridimenzionalni (3-D) kulturi, kjer bi se celice nahajale v okolju, ki je bolj podobno razmeram *in vivo* (178). Diferenciacija in odziv celic na zunanje dejavnike sta v tkivu zaradi drugačne morfologije celic ter njihove adhezije na 3-D ogrodje drugačna kot v klasični celični kulturi (179, 180). Tako 3-D celični modeli predstavljajo vmesni model med živalskim modelom *in vivo* in klasično kulturo *in vitro*.

Prvič se je ideja o 3-D celičnem modelu pojavila v 19. stoletju, ko je angleški fiziolog Sydney Ringer uspel pokazati, da je srce, odvzeto iz živalskega telesa, v raztopini natrija, kalija, kalcija in magnezija še vedno utripalo (181). Danes je znanih že veliko študij na področju biomedicine, ki predstavljajo različne 3-D celične modele za preučevanje celične rasti in mobilnosti (182 - 185), rakavih obolenj (186 - 188) in tkivnega inženiringa (189). Prav tako so znani najpreprostejši 3-D modeli, kot je 3-D celična usedlina, kjer so preučevali obnašanje in diferenciacijo celic (190, 191).

5.1 Celice v tridimenzionalnem celičnem modelu

Glavni površinski receptorji, s katerimi se celica v tkivu povezuje z zunajceličnim ogrodjem so transmembranski proteini, integrini. Integrini so heterodimerne molekule in so sestavljeni iz podenote α in β. Znanih je več kot 20 različnih integrinov, ki jih sestavljajo različne kombinacije α in β podenot. Poleg povezave celice z zunajceličnim ogrodjem imajo integrini tudi pomembno vlogo pri signalizaciji v celici, mobilnosti in uravnavanju celičnega cikla (2).

Vezava integrina na površino je odvisna od komplementarnosti interakcije integrina s substratom, od prisotnosti različnih dvovalentnih kationov in od aktivacije integrina (2).

Integrini se na zunajcelični strani vežejo na specifična kratka aminokislinska ali peptidna zaporedja, ki so značilna za nekatere komponente zunajceličnega ogrodja (kot so kolagen, fibronektin in laminin) (192). Na citosolni strani se podenota β povezuje s

številnimi celičnimi proteini, kot so proteini, ki vežejo podenoto β z aktinskimi filamenti citoskeleta (talin, filamin, F-aktin, miozin, α-aktinin, skelemin), encimi, transkripcijski ko-aktivatorji in številni drugi. Podenota α ima na zunajcelični strani od Ca^{2+} odvisno vezavno mesto, na citosolni strani pa se povezuje z manjšim številom citoplazmatskih proteinov, kot so paksilin, kalretikulin in kaveolin-1 (193, 194).

Poznamo dve vrsti povezav med integrini in citoskeletom (183, 193, 195):

a) fokalni stik − aktinski filamenti citoskeleta se povezujejo preko različnih proteinov (α-aktinin, talin, vinkulin) z podenoto β integrina,
b) hemidezmosom − intermediarni filamenti citoskeleta se povezujejo preko plektina s specifičnim integrinom $\alpha_6\beta_4$. Na tak način se epitelne celice povezujejo z bazalno lamino.

Vezava in komunikacija celice z okoljem je torej zelo kompleksna. Okolje celice vpliva na samo celično obliko in tudi na njen odziv na različne faktorje. Že v zgodnjih 70-ih letih 20. stoletja so znanstveniki opazili morfološke razlike fibroblastov, gojenih na steklu ali vpetih v 3-D kolagenske matrice (196). Mehanizem, s katerim celica zazna dimenzionalno obliko substrata v svojem okolju in razvije primerne adhezine in morfologijo, še ni popolnoma razjasnjen. Prvi stik celice s svojim okoljem je reguliran z hialuronanom. Ko celica začne tvoriti lamelopodije, se na konici le-teh tvorijo fokalni kompleksi, ki predstavljajo predstopnjo fokalnega stika. Ob tvorbi stabilnega fokalnega stika, se $\alpha_5\beta_1$ integrini prenesejo od konice lamelopodija proti centru celice in omogočijo tvorbo fibrilarnega stika. Ta inducira polimerizacijo fibronektina v okolici celice in tako počasi nastaja 3-D okolje, na katerega se celica prilagodi in se s svojim okoljem poveže s 3-D stikom (192).

Tudi migracija celice je kompleksen proces, ki zahteva adhezijo in de-adhezijo celice na njen substrat in se v 2-D in 3-D okolju močno razlikuje (179). S številnimi študijami so skušali pokazati razliko pri gibanju celice v različnih okoljih, ki naj bi bila posledica različne konformacijske strukture molekul zunajceličnega ogrodja (197), izražanja različnih elementov citoskeleta za gibanje celice in odsotnosti adhezinov, ki se vežejo na integrin, kar vodi v ameboidno gibanje (198).

5.2 Vrste tridimenzionalnih celičnih modelov

Številne tehnike in materiali so bili uporabljeni za izdelavo celičnega okolja, ki bi kar najbolje posnemal fizične in kemične lastnosti celičnega okolja v tkivu. 3-D celične modele *in vitro* delimo na dve skupini: 3-D geli in 3-D površine. 3-D gel delno ali v celoti obdaja celico, medtem ko je 3-D površina v stiku s celico le na njeni bazalni strani. Mnoge celice, gojene na 3-D površini, izražajo enake lastnosti kot celice v tkivu. Prednost uporabe 3-D površine je predvsem v tem, da so celice lažje dostopne za različne eksperimente, poleg tega je njihovo opazovanje z mikroskopom lažje. Vendar mnoge celice ne izražajo enakega fenotipa kot v tkivu, če so gojene na 3-D površini, zato je pri njih nujna uporaba 3-D gela (180).

Med najpogosteje uporabljenimi materiali za tvorbo 3-D ogrodja so hidrogeli, ki jih delimo na naravne in umetno pridobljene. Med naravne hidrogele uvrščamo kolagen tipa I in matrigel (bazalni lamini podobna matrica, izolirana iz celic mišjega sarkoma Engelbreth Holm Swarm). Prednost uporabe kolagena tipa I je predvsem v tem, da ga najdemo v naravnem zunajceličnem ogrodju ali bazalni lamini okrog številnih celic, kjer služi kot pomemben mehanski in kemijski dejavnik za pritrditev, migracijo, proliferacijo in diferenciacijo celice. Danes je znanih več različnih pripravkov kolagenov, ki se razlikujejo predvsem v načinu polimerizacije (199 - 203). Matrigel se uporablja predvsem za študijo morfogeneze in diferenciacije celic v 3-D celičnem modelu in je sestavljen iz laminina-1, kolagena IV in vsebuje različne rastne faktorje (202).

Umetno pridobljene hidrogele sestavljajo peptidi, ki so sestavljeni iz zaporedja aminokislin s spreminjajočimi hidrofobnimi in hidrofilnimi stranskimi skupinami in ki so ob dodatku deionizirane vode sposobni polimerizacije v stabilno strukturo β lista (204).

5.3 Delo s tridimenzionalnimi celičnimi modeli

Celice se zelo kompleksno in dinamično odzivajo na svoje okolje. Za morfologijo in metabolizem celice je zelo pomembna interakcija z zunajceličnim ogrodjem. Kljub

temu, da so klasične celične kulture cenejše in je delo z njimi lažje, je na številnih področjih, kot so študija invazivnosti različnih virusov in pridobivanje večjih količin virusnih cepiv (205), preučevanje malignih bolezni (187, 206), preučevanje migracije celic (207) tkivni inženiring (189), nujno delo na 3-D celičnih modelih.

Tudi na področju genske elektrotransfekcije so se pojavili prvi 3-D modeli. Z agregacijo celic so formirali okrogle skupke − celične sferoide, kjer so pokazali uspešno elektropermeabilizacijo z vnosom molekule PI in kalceina (37, 208) ter uspešno gensko transfekcijo z vnosom plazmidne DNA molekule, ki nosi zapis za GFP (37). Avtorji so pokazali, da z električnimi pulzi uspešno vnesejo majhno fluorescentno molekulo PI. Dovajali so deset pulzov, ki so trajali 5 ms z različno jakostjo električnega polja (0.1 kV/cm, 0.3 kV/cm, 0.5 kV/cm) ob prisotnosti molekule PI. Pri jakosti električnega polja 0.3 kV/cm so pokazali uspešen vnos PI v približno 50 % vseh celic v 3-D sferoidu, medtem ko je bil pri jakosti električnega polja 0.5 kV/cm PI uspešno vnesen v praktično vse celice. Z enakimi električnimi parametri so skušali v celice vnesti tudi plazmidno DNA. Pri jakosti električnega polja 0.5 kV/cm so opazili nekaj uspešno transfeciranih celic, vendar le na robu 3-D sferoida, medtem ko z višanjem jakosti električnega polja upade tudi število transfeciranih celic, kar je posledica nižjega preživetja. Slabo učinkovitost genske elektrotransfekcije v 3-D sferoidu so razložili z veliko gostoto celic, ki tako onemogočajo stik DNA s celično membrano, kar je prvi in nujni pogoj za uspešnost metode.

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NAMEN DELA

Namen doktorske disertacije je analizirati vpliv različnih parametrov na učinkovitost genske elektrotransfekcije tako v celični kulturi kot v tridimenzionalnem (3-D) modelu tkiva *in vitro*. Genska elektrotransfekcija z zaključenimi prvimi kliničnimi študijami predstavlja najbolj obetavno alternativo virusni transfekciji na področju genske terapije. Kljub temu pa problem še vedno predstavlja majhna učinkovitost metode v pogojih *in vivo* ter nepoznavanje mehanizmov vnosa DNA v celico z električnimi pulzi. Znano je, da gensko elektrotransfekcijo lahko razdelimo na več korakov: vezava DNA na celično membrano, prenos DNA skozi permeabilizirano membrano, migracija DNA po citoplazmi celice do celičnega jedra, prenos DNA skozi jedrno ovojnico in izražanje gena. Na posamezne korake genske elektrotransfekcije lahko vpliva več različnih dejavnikov. V okviru naše študije se bomo osredotočili na vpliv magnezijevih (Mg^{2+}) ionov na prvi korak genske elektrotransfekcije – vezavo DNA na membrano celice po dovajanju električnih pulzov v celični kulturi *in vitro* – in na morebiten vpliv teh ionov na obstojnost DNA v citoplazmi celice. Na podlagi znanih rezultatov iz literature in novo pridobljenih podatkov bomo skušali razložiti vpliv Mg^{2+} na različne korake vnosa DNA v celico pri genski elektrotransfekciji in s tem skušali izboljšati učinkovitost genske elektrotransfekcije.

V bolj kompleksnih celičnih sistemih, kot so tkiva in organi, na učinkovitost genske elektrotransfekcije vpliva predvsem struktura tkiva. Do danes še nihče ni analiziral genske elektrotransfekcije v 3-D modelu *in vitro,* zato bomo skušali razviti preprost 3-D model *in vitro*, kjer bomo pokazali možnost analize vpliva različnih parametrov električnih pulzov na učinkovitost metode. Takšen model bi lahko omogočal študijo optimizacije protokolov in razumevanje mehanizmov genske elektrotransfekcije *in vitro* ter pripomogel k zmanjšanju številnih eksperimentov *in vivo* in s tem potrebo po velikem številu žrtvovanih živali.

Skeletne mišice predstavljajo zanimiv ciljni organ za vnos genov z gensko elektrotransfekcijo, ker so lahko dostopne, električno dobro prevodne in dobro prekrvavljene. Izražanje genov v mišičnih celicah *in vivo,* vnesenih z dovajanjem električnih pulzov, je daljše in učinkovitejše v primerjavi z izražanjem genov, vnesenih z drugimi metodami. Zato bomo primerjali učinkovitost genske elektrotransfekcije z

lipofekcijo na primarnih mišičnih celicah. Analiza *in vitro* različnih parametrov genske elektrotransfekcije na primarnih celičnih kulturah in teoretična obravnava le-teh lahko pripomoreta k razlagi mehanizmov in izboljšanju metode *in vivo*.

UPORABLJENE METODE

1. Izolacija plazmidne DNA iz DH5α bakterijskega seva *Escherichia coli* s komercialnim izolacijskim kompletom.
2. Spektrofotometrija za določanje koncentracije plazmidne DNA.
3. Agarozna gelska elektroforeza za določanje uspešnosti izolacije in velikosti plazmidne DNA.
4. Elektroporacija.
   - 4.1 Vnos majhne molekule PI v celice
   - 4.2 Vnos plazmidne DNA v celice
5. Spektrofluorometrija.
   - 5.1 Določanje uspešnosti elektropermeabilizacije (vnosa PI v celice)
   - 5.2 Določanje celične viabilnosti
6. Pretočna citometrija.
   - 6.1 Določanje uspešnosti genske elektrotransfekcije (vnosa plazmidne DNA, ki nosi zapis za GFP)
7. Fluorescentna invertna mikroskopija.
   - 7.1 Določanje uspešnosti elektropermeabilizacije (vnosa PI v celice)
   - 7.2 Določanje uspešnosti genske elektrotransfekcije (vnosa plazmidne DNA, ki nosi zapis za GFP)
8. Merjenje celične viabilnosti z metodo CVDE

REZULTATI IN RAZPRAVA

STRUKTURA DOKTORSKE DISERTACIJE

Doktorska disertacija je sestavljena iz petih poglavij, ki jih sestavljajo znanstveno-raziskovalni članki (od tega sta dva objavljena in dva v pripravi za objavo), ter prispevek predstavljen na konferenci. K **Poglavju 3** smo priključili **Dodatek k poglavju** z namenom predstavitve rezultatov, ki v članku niso bili objavljeni.

V **Poglavju 1** bomo predstavili vpliv koncentracije Mg^{2+} ionov v elektroporacijskem mediju na uspešnost elektropermeabilizacije in genske elektrotransfekcije ter vpliv na preživetje celic. Naš namen bo izboljšati učinkovitost genske elektrotransfekcije, zato bomo uporabili elektroporacijske medije z različno koncentracijo Mg^{2+} ionov. Uspešna elektropermeabilizacija celične membrane in metabolno aktivna živa celica sta nujna pogoja za učinkovit prenos DNA v celico z električnimi pulzi in izražanje vnesenega gena. Za uspešno gensko elektrotransfekcijo je poleg elektropermeabilizirane membrane nujna tudi interakcija DNA s celično membrano. Na slednjo naj bi imeli velik vpliv Mg^{2+} ioni, saj naj bi pripomogli k zmanjšanju elektrostatske odbojne sile med negativno nabito DNA in negativno nabito celično membrano in tako povečali uspešnost genske elektrotransfekcije. Učinkovitost elektropermeabilizacije celične membrane bomo določali z detekcijo vnosa majhne molekule propidijevega jodida (PI), učinkovitost genske elektrotransfekcije z vnosom plazmidne DNA, ki nosi zapis za reporterski gen za zeleno fluorescirajoči protein (GFP), preživetje celic pa z barvilom kristal vijolično. Pokazali bomo, da Mg^{2+} ioni privedejo do višjega vnosa propidijevega jodida s pomočjo električnih pulzov in višjega preživetja celic, medtem ko je učinkovitost genske elektrotransfekcije občutno znižana. Zato bomo preučili vpliv Mg^{2+} ionov na interakcijo s fluorescentnim barvilom TOTO-1 obarvane DNA s celično membrano po dovajanju električnih pulzov.

V **Poglavju 2** bomo nadaljevali raziskavo in skušali pokazati vpliv koncentracije Mg^{2+} ionov na moč vezave DNA na celično membrano po dovajanju električnih pulzov. Ker je DNA polianionska molekula, nanjo v električnem polju deluje elektroforetska sila, zato bomo z obračanjem smeri električnih pulzov pri različnih koncentracijah Mg^{2+} ionov opazovali interakcijo s TOTO-1 obarvane DNA s celično membrano. Mg^{2+} ioni imajo prav tako lahko tudi vpliv na prenos DNA po citoplazmi celice, saj znotrajcelične DNaze, aktivirane z Mg^{2+} ioni, lahko razgradijo plazmidno DNA in tako vplivajo na

učinkovitost genske elektrotransfekcije. Zato bomo v nadaljevanju predstavili vpliv DNaznega inhibitorja na učinkovitost genske elektrotransfekcije pri različnih koncentracijah Mg^{2+} ionov.

V **Poglavju 3** bomo predstavili uspešno izdelan, preprost 3-D model tkiva *in vitro* za študijo elektropermeabilizacije in genske elektrotransfekcije. Celica v celični kulturi je drugačne morfologije in se drugače odziva na zunanje dejavnike kot celica v tkivu. Poleg tega je mobilnost DNA v tkivu močno zmanjšana zaradi zunajceličnega ogrodja, ki obdaja celice. Zato se rezultati genske elektrotransfekcije, pridobljeni v kulturi *in vitro*, ne skladajo vedno z rezultati, pridobljenimi v okolju *in vivo*. Danes poznamo različne 3-D modele *in vitro*, s katerimi skušajo tako gojenim celicam približati okolje *in vivo*. Naš namen bo zgraditi preprost 3-D model, kjer bodo celice vpete med kolagenska vlakna in bodo tako gojene sposobne preživeti dalj časa. Z uporabo električnih pulzov bomo pokazali uspešen vnos majhnih molekul (PI) ter plazmidne DNA v tako gojene celice in analizirali vpliv različnih koncentracij plazmidne DNA in dolžine električnega pulza na učinkovitost genske elektrotransfekcije.

V **Dodatku k poglavju 3** bomo predstavili različne (tudi negativne) poizkuse izdelave 3-D modela tkiva *in vitro* za študijo genske elektrotransfekcije, predstavljenega v Poglavju 3. Prikazali bomo več različnih 3-D ogrodij za celice, kjer bomo najprej testirali preživetje različnih celičnih linij in nato uspešnost genske elektrotransfekcije.

V **Poglavju 4** bomo predstavili analizo vpliva mobilnosti DNA na učinkovitost genske elektrotransfekcije v 3-D modelu tkiva *in vitro*. Glavni problem uspešnosti genske elektrotransfekcije *in vivo* namreč predstavlja slaba mobilnost DNA v tkivu. Zaradi kompleksne strukture in gostote zunajceličnega ogrodja, ki obdaja celice v tkivu, je namreč po dovajanju električnih pulzov na celično membrano vezane manj DNA in je zato posledično zmanjšana učinkovitost genske elektrotransfekcije. Z uporabo različnih naborov električnih parametrov in različnega načina aplikacije DNA bomo skušali izboljšati transfekcijo gena v 3-D modelu *in vitro*. Obračali bomo smeri električnih pulzov in uporabljali samo visokonapetostne pulze, samo nizkonapetostne pulze ali pa kombinacijo obojih. Pokazali bomo, da primerna izbira kombinacije visokonapetostnega in nizkonapetostnega pulza lahko omogoča večjo uspešnost genske

elektrotransfekcije v 3-D modelu *in vitro*, kot dovajanje izključno visokonapetostnega pulza.

V zadnjem delu doktorske naloge (**Poglavje 5**) bomo primerjali dve metodi vnosa DNA *in vitro* (lipofekcijo in elektroporacijo) v primarne humane mišične celice, pridobljene iz mišičnega tkiva, odvzetega pri ortopedskih operacijah. S številnimi raziskavami *in vivo* so pokazali, da je mišično tkivo pomembno ciljno tkivo za vnos terapevtskih genov z dovajanjem električnih pulzov, saj produkti le-teh delujejo lokalno in sistemsko. Z uporabo različnih parametrov električnih pulzov bomo skušali optimizirati gensko elektrotransfekcijo in preživetje celic za primarno celično linijo. Pokazali bomo, da je učinkovitost vnosa DNA z lipofekcijo in elektroporacijo primerljiva, medtem ko je preživetje celic po dovajanju električnih pulzov občutno znižano.

Poglavje 1

Vpliv magnezijevih ionov na uspešnost genske elektrotransfekcije in elektropermeabilizacije

Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization

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POVZETEK

Genska elektrotransfekcija je obetavna nevirusna metoda, ki z dovajanjem električnih pulzov omogoča vnos DNA v celice. Na uspešnost posameznih korakov genske elektrotransfekcije vplivajo številni parametri. Interakcija DNA s celično membrano predstavlja enega izmed korakov genske elektrotransfekcije, na katerega imajo lahko vpliv divalentni kationi v elektroporacijskem mediju. Predstavili smo vpliv različnih koncentracij Mg^{2+} ionov v elektroporacijskem mediju na učinkovitost vnosa majhnih molekul (propidijevega jodida) in DNA v celice z dovajanjem električnih pulzov ter vpliv Mg^{2+} ionov na preživetje celic. Prav tako smo s pomočjo barvila TOTO-1 prikazali interakcijo DNA s celično membrano pri različnih koncentracijah Mg^{2+}. Naša študija je pokazala, da višje koncentracije Mg^{2+} ionov pripomorejo k višjemu vnosu propidijevega jodida in k višjemu preživetju celic, medtem ko je genska elektrotransfekcija znižana. Ker smo opazili večjo količino s TOTO-1 obarvane DNA na celični membrani pri uporabi višje koncentracije Mg^{2+} ionov, predlagamo, da ti ioni po dovajanju električnih pulzov vežejo DNA na celično membrano s tako intenziteto, da ta ne more prehajati skoznjo, kar privede do znižanja uspešnosti genske elektrotransfekcije. Mg^{2+} ioni pa imajo lahko vpliv tudi na druge korake genske elektrotransfekcije. Naši rezultati prav tako kažejo, da le prepustnost membrane ni dovolj za učinkovito gensko elektrotransfekcijo.

Ključne besede: Mg^{2+} ioni, genska elektrotransfekcija, elektropermeabilizacija, divalentni kationi, TOTO-1.

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Short communication

Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization

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ABSTRACT

Gene electrotransfer is a promising nonviral method that enables DNA to be transferred into living cells with electric pulses. However, there are many parameters that determine gene electrotransfer efficiency. One of the steps involved in gene electrotransfer is interaction of DNA with the cell membrane. Divalent cations in the electroporative media can influence the anchoring of DNA to the cell membrane and by that gene electrotransfer efficiency. Here we report the effect of different concentrations of Mg^{2+} on electropermeabilization for small molecule (propidium iodide), gene electrotransfer and viability of the cells. We also used TOTO-1 dye to visualize DNA-cell membrane interaction for different [Mg]. For this purpose, we used different electroporative media with increasing [Mg]. Our study shows that higher [Mg] lead to higher electropermeabilization for propidium iodide and higher viability, while causing lower gene electrotransfer efficiency. Because we observed higher TOTO-1 labeled DNA at cell surface when using higher [Mg], we suggest that Mg^{2+} ions can bind DNA at cell surface at such strength that cannot pass into the cell during application of electric pulses, which can lead to lower gene transfection. There may also be other mechanisms involved, since there are many steps of gene electrotransfer on which Mg^{2+} ions can have an effect on. Our results also imply that membrane permeability changes are not sufficient for an efficient gene electrotransfer.

1. Introduction

Exposing cells to short intense electric pulses increases permeability of cell membrane. It has been shown on lipid membrane model that electric field hastily reorients lipid heads in the membrane and that the number of local spots with fluid conformation increases [1]. The phenomenon is shown as electroporation or electropermeabilization. It has been used for many years to introduce small molecules that otherwise cannot pass the cell membrane into cells [2–4]. In 1982 Neumann with colleagues first achieved successful transfection of a foreign gene into eukaryotic cells with electric pulses [5,6]. Even though today gene electrotransfer is widely used to transfect all types of cells and represents a safer alternative to viral vectors, the processes underlying transfer of genetic material through cell membrane and into the cell are still not completely understood [7,8].

Different mechanisms of electropermeabilization for small molecules have been reported [9–14]. In addition, gene electrotransfer mechanisms are also available in the literature which suggests that several steps are involved in gene electrotransfer [10,13,15–21]: (a) formation of a complex between DNA and cell membrane, (b) translocation of DNA across the permeabilized membrane, (c) transfer of DNA from cytoplasm into nucleus and (d) gene expression.

The main obstacle in gene electrotransfer of mammalian cells is its low efficiency, which depends not only on permeability changes of cell membrane but also on the way DNA interacts with the membrane and migrates towards the nucleus. Although several studies showed that different parameters (e.g. cell type, temperature, parameters of electric pulses, ions in electroporative media) have influence on the efficiency of uptake of small molecules as well as on gene electrotransfer [10,22–39], it was also suggested that one of the key parameters which affect the process of DNA interaction with the cell membrane is the concentration of ions in media [15,17,40]. It was suggested that especially divalent cations (such as Ca^{2+} or Mg^{2+}) may have important impact on forming a complex between DNA and the cell membrane during application of electrical pulses, which can lead to the improvement of gene electrotransfer [17,29,40]. Namely, since DNA is negatively charged polyelectrolyte, divalent cations can bridge the DNA with negatively charged cell membrane during application of electric pulses. This hypothesis was supported by the study of anionic unilamellar vesicles, where DNA adsorption to vesicle membrane was greatly enhanced with increasing concentration of divalent cations such as Ca^{2+} ions [41,42].

Up to now only few researchers have experimentally investigated the effect of different ions on gene electrotransfer efficiency in vitro or in vivo [5,6,17,28,29,32,40], however their results are contradictory.

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In one of the in vitro studies researches observed increased gene electrotransfer efficiency for increased [Mg] from 1 to 8 mM [17]. However, this is not in agreement with Neumann et al. [5,6] study. This can be a consequence of small DNA concentration used by Xie and Tsong [17], which resulted in a relatively low percentage of transfection (max. 9%) and therefore higher [Mg] could have a positive effect at such low plasmid concentration.

In in vivo studies the influence of a wide range of ionic composition in electroporative media on gene electrotransfer was also analyzed. It was demonstrated that gene expression decreased for higher concentrations of ions in used media [28]. Further some researchers showed that higher concentration of divalent cations limits plasmid DNA entrance into the cell during electroporation [32]. They proposed that higher concentration of divalent cations alters stability and physical properties of DNA molecules. However, in another in vivo study it was shown, that gene electrotransfer efficiency in mice is improved by increasing the concentrations of ions in initial injected media [29].

Hence, we report here a study of the influence of different [Mg] in electroporative media on electropermeabilization for propidium iodide (PI), cell viability and gene electrotransfer. The main objective of our study was to understand the role of Mg^{2+} ions on different steps of gene electrotransfer by analyzing separately effects of Mg^{2+} ions on electropermeabilization for PI, viability and transfection.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary cells (CHO-K1) were grown in Ham's tissue culture medium for mammalian cells with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37 °C in a humidified 5% CO_2 atmosphere in the incubator (Kambič, Slovenia).

Electroporation was performed on 24 hour old cell culture in different electroporative media.

2.2. Plasmid DNA

Plasmid pEGFP-N_1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in DH5α strain of Escherichia coli and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

2.3. Electropermeabilization for propidium iodide

To evaluate electropermeabilization for propidium iodide of CHO cells in different electroporative media with different concentration of Mg^{2+} ([Mg] = 1, 4, 10 and 50 mM), propidium iodide (PI) was used. PI is a small molecule which enters a cell, if the membrane of the cell is permeabilized [43]. All electroporative media were isoosmolar (10 mM phosphate buffer, NaH_2PO_4/Na_2HPO_4, pH = 7.4), where media with [Mg] = 1 mM represents standard electroporative media.

Cell suspension was prepared by 0.25% trypsin/EDTA solution (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), centrifuged for 5 min at 1000 rpm (180 × *g*) at 4 °C (Sigma, Germany) and resuspended in different electroporative media to a cell density of $\rho = 2.5 \times 10^6$ cells/ml. Because of easier survey of the results we used only electroporative media with [Mg] = 1 mM, 4 mM, 10 mM and 50 mM. For electroporation, cuvettes with built in aluminium electrodes were used (Eppendorf, Hamburg, Germany).

The volume of the cells placed in cuvette was 200 µl (for each electric pulse parameter). Immediately before electric pulse application, 2 µl of 0.15 mM PI was added to the media. Final concentration of PI in a sample was 10 µg/ml.

Samples were then exposed to electric pulses to deliver PI into the cells using Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy) pulse generator. A train of four rectangular pulses with duration of 200 µs and repetition frequency 1 Hz was applied. The applied voltages were 240 V, 400 V and 560 V which resulted in 0.6 kV/cm, 1.0 kV/cm and 1.4 kV/cm *E*, respectively. Applied electric field is defined by

$$E = U(\text{appl}) / d, \tag{1}$$

where *U*(appl) denotes applied voltage and *d* electrode distance ($d = 4$ mm). To achieve saturation of fluorescence of PI in cells we also exposed cells to $E = 1.8$ kV/cm. No electric pulses were applied to cells in control.

After pulses were applied, cells were incubated for 3 min at room temperature (22 °C) and then centrifuged for 5 min at 1000 rpm (180 × *g*) at 4 °C to remove extracellular PI that did not enter the cells. 200 µl of fresh media was added and the uptake of PI was evaluated with spectrofluorometer (Tecan infinite M200, Tecan Austria GmbH) at 617 nm. The permeabilization (uptake of PI) is defined as:

$$\text{Permeabilization } (\%) = \frac{F_{(\text{PI},E)} - F_{(\text{PI},E=0)}}{F_{(\text{PI},\max)} - F_{(\text{PI},E=0)}} \cdot 100, \tag{2}$$

where $F_{(\text{PI},E)}$ denotes fluorescence intensity of cells subjected to electric pulses, $F_{(\text{PI},E=0)}$ fluorescence intensity of cells at $E = 0$, i.e. cells in control, and $F_{(\text{PI},\max)}$ fluorescence intensity of cells at $E = 1.8$ kV/cm, i.e. where saturation fluorescence is achieved.

2.4. Cell viability

Cell viability was evaluated with crystal violet dye elution method (CVDE) as previously described [44]. After exposing cells to electric pulses with plasmid DNA in concentration 40 µg/ml, the cells were plated in multiwells at a cell density of $\rho = 1 \times 10^5$ cells/ml and grown for 24 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere in the incubator. Because of easier survey of the results we used only electroporative media with [Mg] = 1, 4, 10 and 50 mM.

After 24 h samples were stained with 0.1% crystal violet (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution in sodium phosphate media (10 mM phosphate buffer, NaH_2PO_4/Na_2HPO_4, pH = 7.4) for 30 min at room temperature (22 °C). After incubation, crystal violet was removed and cells were washed with sodium phosphate media and lysed with 10% acetic acid. The same staining protocol with crystal violet was performed also in wells without cells (background wells). CVDE is a simple assay that evaluates cell density by staining DNA. After elimination of the excess dye, the absorbance at 540 nm was measured with spectrofluorometer (Tecan infinite M200, Tecan Austria GmbH), which is proportional to the amount of viable cells in the well.

The experimental fraction of cells stained with crystal violet (CV) is defined as:

$$f(\text{CV}) = \frac{A_{(\text{CV},E)} - A_{(\text{CV},\text{bg})}}{A_{(\text{CV},E=0)} - A_{(\text{CV},\text{bg})}}, \tag{3}$$

where $A_{(\text{CV},E)}$ denotes absorbance of cells subjected to electric pulses, $A_{(\text{CV},\text{bg})}$ background absorbance and $A_{(\text{CV},E=0)}$ absorbance of cells at $E = 0$, i.e. that of non-pulsed cells.

The fraction of stained cells equals cell viability, therefore $f(\text{CV}) = f(\text{viability})$ and:

$$\text{Viability } (\%) = f(\text{CV}) \cdot 100. \tag{4}$$

2.5. Gene electrotransfer

Electroporation was performed on CHO cells that were in the exponential growth phase. Cell suspension was prepared in the same

way as for electropermeabilization for PI using different electroporative media.

Plasmid DNA was added to cell suspension in concentration 40 μg/ml. After 2–3 min incubation of DNA with CHO cells at room temperature (22 °C), samples were exposed to square wave electric pulses using Cliniporator™ pulse generator. For gene electrotransfer the same electric pulse protocol was used as for electropermeabilization for PI. After exposing cells to electric pulses, fetal calf serum (FCS-Sigma, USA) was added (25% of sample volume) to preserve cell viability. Cells were then incubated for 5 min at 37 °C to allow cell membrane resealing and then grown for 24 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere in the incubator. After 24 h cells were trypsinized, centrifuged for 5 min at 1000 rpm (180×*g*) at 4 °C and resuspended in phosphate buffered saline (PBS) to a cell density of $\rho = 1 \times 10^6$ cells/ml. The samples were analyzed with Coulter EPICS Altra flow cytometer (Beckman Coulter Electronics) equipped with a laser emitting at 509 nm. 9000 events were recorded. As a representative case, histograms obtained from the flow cytometer and scatter plots are displayed in Fig. 1. Gene electrotransfer efficiency was determined by measuring fraction of cells expressing green fluorescent protein:

$$f(GFP) = N_{GFP} / N, \tag{5}$$

where $N_{(GFP)}$ denotes number of viable fluorescent cells and N number of all viable cells in a given sample. The fraction of cells expressing green fluorescent protein equals fraction of transfected cells for the given conditions, $f(\text{GFP}) = f(\text{transfection})$, therefore

$$\text{Transfection } (\%) = f(GFP) \cdot 100. \tag{6}$$

All experiments were repeated three times on different days. Results from different repetitions of experiments were pooled together and are presented as mean and standard deviation of the mean.

2.6. Visualization of DNA-cell membrane interaction for different [Mg]

To visualize DNA interaction with cell membrane TOTO-1 (Molecular Probes — Invitrogen, Carlsbad, California, USA) nucleic acid stain was used which was already shown to enable direct visualization of DNA with the cell membrane [45]. The plasmid pEGFP-N_1 was labeled with 2.3×10^{-4} M TOTO-1 DNA intercalating dye for 60 min on ice. Plasmid concentration was 1 μg/μl, which yields an average base pair to dye ratio of 5.

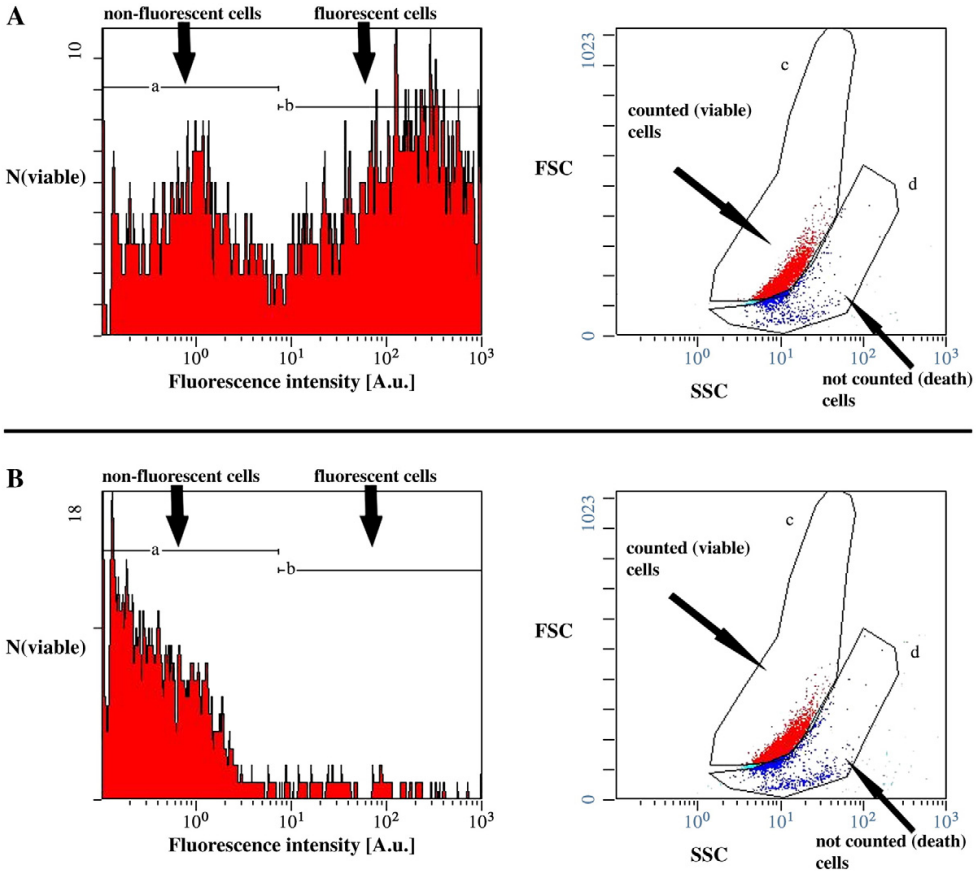

Fig. 1. Representative histograms for GFP fluorescence (left) and scatter plots (right) obtained from the flow cytometer for (A) [Mg] = 0.5 mM and (B) [Mg] = 50 mM. Fraction of cells expressing GFP was calculated as a number of viable fluorescent cells divided by number of all viable cells (see Eqs. (5) and (6)). Histograms for GFP fluorescence (left) shows in region (a) counted viable non-fluorescent cells and in region (b) counted viable fluorescent cells. Scatter plots (right) shows in region (c) counted (viable) cells and in region (d) not counted (death) cells. 4×200 μs pulses ($E = 1.4$ kV/cm) with repetition frequency 1 Hz were used at room temperature ($T = 22$ °C).

CHO-K1 cells were plated as a monolayer culture in Labtech chamber for 1 h in cell culture medium at 37 °C in a humidified 5% CO_2 atmosphere in the incubator at cell density of $\rho = 1 \times 10^5$ cells/ml. After 1 h culture medium was removed and replaced with electroporative media with different concentration of Mg^{2+} ([Mg] = 1 mM and 50 mM). Immediately before electric pulse application, labeled plasmid was added to cells in concentration 10 μg/ml. Samples were then exposed to electric pulses using Jouan GHT 1287 electroporator (Jouan, St. Herblain, France). An oscilloscope Wave surfer™ 422 (Le croy, Chestnut Ridge, New York, USA) monitored pulse shape. A train of eight pulses with duration of 5 ms and repetition frequency 1 Hz was applied ($E = 0.7$ kV/cm). The distance between a pair of two wire stainless steel parallel electrodes was $d = 2$ mm.

To monitor the interaction of DNA with the cell membrane fluorescent microscopy (Zeiss 200, Axiovert, ZR Germany) was used with 100× oil immersion objective. The images (see Fig. 3) were recorded using imaging system (MetaMorph imaging system, Visitron, ZR Germany).

3. Results

Main objective of our study was to determine the role of Mg^{2+} ions on DNA adsorption on cell surface by means of electric pulses, to analyze separately different effect of these ions on electropermeabilization for PI, viability and gene electrotransfer and to determine whether higher [Mg] improves gene electrotransfer efficiency.

The effect of [Mg] was followed by determining the uptake of PI (permeabilization), the fraction of viable cells (viability) and fraction of cells expressing green fluorescent protein (transfection). The electroporation protocol was the same in all experiments: applied voltages were 240 V, 400 V and 560 V which resulted in electric field strengths 0.6 kV/cm, 1 kV/cm and 1.4 kV/cm; four pulses with pulse duration 200 μs and repetition frequency 1 Hz were used.

Fig. 2A shows the percentage of permeabilization for PI, which represent the uptake of PI for different electric field strength (E) as a function of different Mg^{2+} concentrations in electroporative media. Increasing electric field strength results in an increase in electropermeabilization efficiency for PI irrespective of the electroporative media used. Increasing the [Mg] resulted in increasing electropermeabilization efficiency for PI.

We also tested the viability of the cells in different media. The increase of the electric field strength was followed by the decrease of the number of viable cells in all media (Fig. 2B). The highest viability was observed in media with [Mg] = 1 mM at $E = 0.6$ kV/cm, where approximately 83% of cells survived. At higher electric field strengths above the electroporation threshold (1.0 kV/cm and 1.4 kV/cm) we however obtained higher viability with higher [Mg]. Our results therefore showed that increasing [Mg] in electroporative media results in better viability of electroporated cells.

In Table 1 percentage of transfection, which represent the gene electrotransfer efficiency is shown with different [Mg] in electroporative media for different E. In general, gene electrotransfer efficiency increased with increasing E. At [Mg] = 0.5 mM we consistently observed the highest gene electrotransfer at each field. Maximum gene expression was observed at highest E (1.4 kV/cm) where approximately more than 50% of cells were expressing GFP gene similarly as in our previous study [34]. Further increase in [Mg] (from 1 to 50 mM) resulted in a decrease of gene expression at each field, contrary to what was observed for electropermeabilization for PI. The percentage of transfection in electroporative media with [Mg] = 50 mM was the lowest, where even at $E = 1.4$ kV/cm only 13% of transfected cells were obtained. Altogether the gene electrotransfer efficiency in electroporative media with [Mg] = 50 mM dropped to less than a half in comparison with gene electrotransfer efficiency in electroporative media with only [Mg] = 0.5 mM.

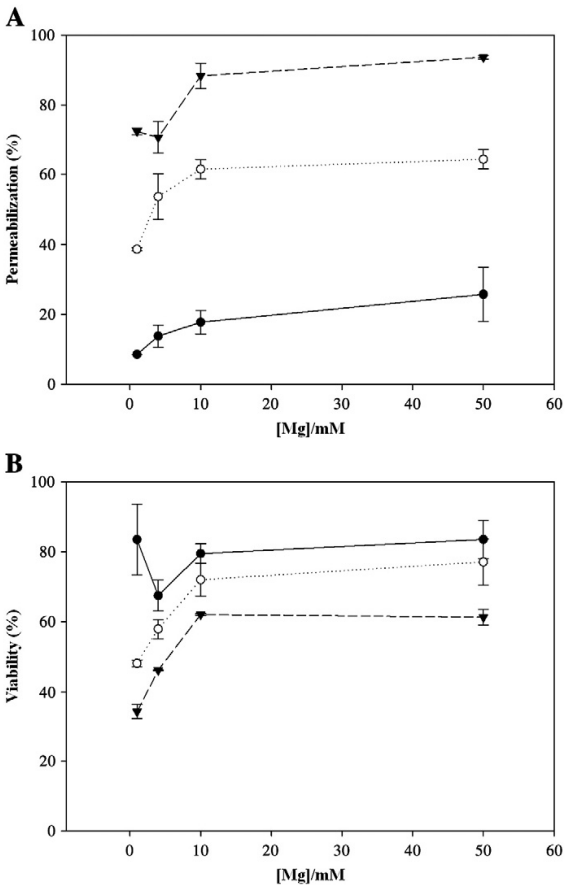

Fig. 2. Effect of Mg^{2+} ions on (A) cell electropermeabilization for PI and on (B) cell viability. (A) the percentage of permeabilization (uptake of PI – see Eq. (2)) and (B) the percentage of viability (see Eqs. (3) and (4)) as a function of different [Mg] in electroporative media (1, 4, 10 and 50 mM) for different electric field strengths: (●) 0.6 kV/cm; (○) 1.0 kV/cm and (▼) 1.4 kV/cm is shown; 4×200 μs pulses with repetition frequency 1 Hz were used at room temperature ($T = 22$ °C). Cell density $\rho = 2.5 \times 10^6$ cells/ml. Values represent means ± standard deviation.

To understand if the decrease in gene electrotransfer efficiency when using higher [Mg] is a consequence of intense binding of DNA at cell membrane, visualization of DNA interaction with membrane was performed by using TOTO-1 labeled plasmid DNA. The labeled plasmid was added to cells and when electric pulses were applied, accumulation of the labeled DNA at the cell membrane was observed,

Table 1
Effect of Mg^{2+} ions on gene electrotransfer. The percentage of transfected cells (%) – cells expressing GFP (see Eqs. (5) and (6)) in electroporative media with different [Mg] is presented for different electric field strengths. Other conditions are as in the caption of Fig. 2. Values represent means ± standard deviation.

Transfection (%)	0.6 kV/cm	1.0 kV/cm	1.4 kV/cm
0.5 mM [Mg]	7.1(±0.3)	41.1(±0.3)	53.3(±0.9)
1 mM [Mg]	5.7(±0.9)	37.2(±4.0)	50.7(±5.8)
2 mM [Mg]	5.3(±1.0)	31.1(±3.5)	46.7(±4.7)
4 mM [Mg]	4.2(±0.6)	28.2(±2.7)	40.6(±4.4)
6 mM [Mg]	3.5(±0.4)	23.8(±0.6)	34.9(±0.9)
8 mM [Mg]	3.0(±0.3)	20.0(±0.3)	30.8(±1.2)
10 mM [Mg]	3.5(±0.2)	20.7(±1.2)	32.7(±1.1)
50 mM [Mg]	1.5(±0.1)	8.3(±0.6)	13.2(±1.0)

as was already shown before [45–47]. No fluorescence caused by spontaneous adsorption of DNA to the plasma membrane was detected when no pulses were applied (control sample - data not shown). When using [Mg] = 1 mM, localized fluorescence spots were observed only on the membrane side facing the cathode (see Fig. 3B). Increase in concentration of Mg^{2+} ions to 50 mM resulted in an increase of fluorescence intensity of accumulated DNA at whole cell membrane surface (Fig. 3D). We repeated the experiment three times and this effect was always observed.

4. Discussion and conclusions

Transfer of DNA into the cells by membrane electroporation is an established method for gene delivery both in vitro and in vivo. The main problem in gene electrotransfer of mammalian cells in vivo is however it's relatively low efficiency. In some of the reports researchers emphasize an importance of cations on gene electrotransfer efficiency and suggest that divalent cations enhance adsorption of negatively charged DNA onto negatively charged cell membrane (bridging effect) which could improve gene electrotransfer efficiency [17,40].

Since Mg^{2+} ion is one of the most common divalent cation used in standard electroporative media, the aim of our study was to evaluate the effects of various Mg^{2+} concentrations on cell electropermeabilization for PI, viability and gene electrotransfer in order to better understand mechanism of gene electrotransfer (specially the formation of a complex between DNA and cell membrane during electric pulses), and furthermore to determine whether higher [Mg] can improve gene electrotransfer.

Our results show that higher [Mg] in electroporative media increase electropermeabilization for PI as shown in Fig. 2A. This is in agreement with other studies which showed that changing ion concentration in the media can have an effect on reorganization of membrane lipid bilayer, which can result in a higher uptake of small molecules such as PI [48].

To determine the influence of [Mg] on gene electrotransfer efficiency, we performed gene electrotransfer with plasmid DNA encoding GFP in different media. In general transfection efficiency decreased with increasing [Mg] for all electric filed strengths (see Table 1). We also tested gene electrotransfer efficiency in media with even lower concentrations of Mg^{2+} ([Mg] = 0.5 mM) with the same pulse parameters as for other media. The percentage of cells expressing GFP gene was higher than in standard media with [Mg] = 1 mM.

In order to directly analyze the effect of Mg^{2+} concentrations on the formation of a complex between DNA and cell membrane, we performed visualization of DNA interaction with the cell membrane using TOTO-1 nucleic acid stain (Fig. 3). As was already shown by others [45–47], plasmid interacted with the membrane by forming localized spots. Although gene electrotransfer efficiency decreased for higher [Mg], it appears that quantity of accumulated DNA at the cell membrane increased with increasing [Mg]. It was already proven that Mg^{2+} ions facilitate the absorption of DNA at the cell surface and are therefore necessary for successful gene electrotransfer [6]. But maybe at some point Mg^{2+} at higher concentrations bind DNA at the cell membrane at such intensity, that DNA cannot cross the membrane during electroporation.

However, since formation of a complex between DNA and cell membrane is only one step necessary for successful gene electrotransfer, the decrease in transfection efficiency for increasing [Mg] could be also a consequence of other factors. Namely, divalent cations such as Mg^{2+} ions interact with plasmid DNA and can alter the stability and topology of DNA molecule and therefore can induce structural transition of a DNA into a compact form [49]. It was shown by some researchers that ions strongly affect biologically significant behavior of DNA in the cell, such as transcriptional initiation and elongation [46], which can consequently result in decreased gene expression. It was also shown that for some conditions divalent ions

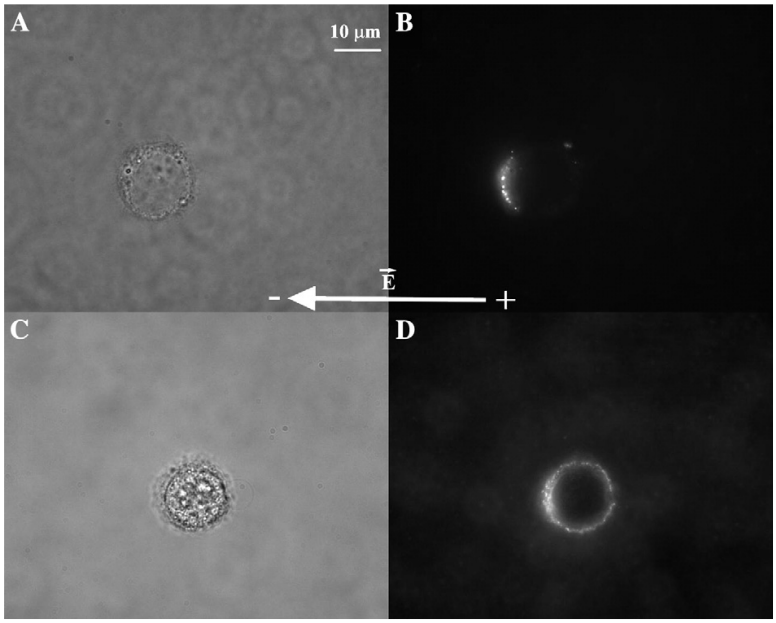

Fig. 3. Fluorescence microscopy observation of DNA-membrane interaction when using different [Mg]. Plated cells were incubated in presence of TOTO-1 labeled DNA (pEGFP-N_1) and different [Mg] in electroporative media (1 mM and 50 mM). 8×5 ms (E = 0.7 kV/cm) pulses were applied with repetition frequency of 1 Hz to observe DNA–membrane interaction (concentration of labeled DNA in electroporative media was 10 μg/ml). (A) phase contrast image of treated cells in [Mg] = 1 mM; (B) fluorescence image of treated cells in [Mg] = 1 mM; (C) phase contrast image of treated cells in [Mg] = 50 mM; (D) fluorescence image of treated cells in [Mg] = 50 mM. The white arrow in the middle indicates the direction of the applied electric field.

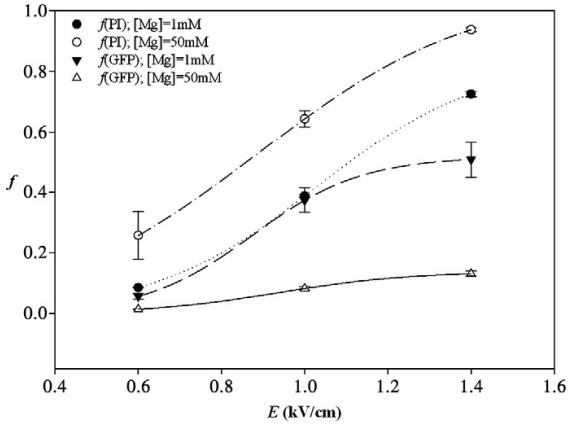

Fig. 4. Correlation between electropermeabilization for PI and gene electrotransfer at [Mg] 1 mM and 50 mM. Uptake of PI - f(PI) = Permeabilization/100 (see Eq. (2)) and fraction of cells expressing green fluorescent protein - f(GFP) (see Eq. (5)) as a function of different electric filed strength used is presented: (●) f(PI) in [Mg] = 1 mM; (○) f(PI) in [Mg] = 50 mM; (▼) f(GFP) in [Mg] = 1 mM; (△) f(GFP) in [Mg] = 50 mM. Values represent means ± standard deviation.

(mostly Mg^{2+}) increase activity of DNAse enzyme, which as a result can decrease the gene electrotransfer efficiency [50], in electroporative media with high concentration of added Mg^{2+} ions.

Our analyses of correlation between electropermeabilization for PI and gene electrotransfer in combination with Mg^{2+} ions led us to the conclusion that although electroporation is crucial for transfection, gene electrotransfer efficacy is not strictly correlated to permeability changes in cell membrane. This is also in agreement with other authors, where they also obtained that gene electrotransfer is a more complex process than classical electroporation [25] and that several steps are involved. From Fig. 4 it is clearly apparent, that generally at a given E, f(GFP) i.e. transfection for DNA is fraction-wise different from f(PI) i.e. permeabilization/100 for PI and that permeabilization of cells for PI is not the same as permeabilization for DNA.

In order to separate different effects of Mg^{2+} ions on gene electrotransfer efficiency, we tested whether Mg^{2+} inhibitory effect on gene electrotransfer is a result of lower cell viability. We obtained, that increasing [Mg] in electroporative media results in higher viability of electroporated cells, which can be explained that addition of Mg^{2+} ions improves the recovery of electrolyte homeostasis as has been already shown in vivo [51].

In conclusion, we have shown that cell electropermeabilization for PI, gene electrotransfer efficiency and cell viability depend on the concentration of Mg ions in electroporative media. While electropermeabilization for PI and viability increases with higher [Mg], gene electrotransfer decreases. Because of the opposite effect of Mg^{2+} ions on cell electropermeabilization for PI and on gene electrotransfer efficiency, we can conclude that membrane permeabilization is necessary but not sufficient for an efficient gene transfer as already shown by other study [25]. We suggest that Mg^{2+} ions at higher concentrations bind DNA to the cell membrane strong enough to prevent translocation of DNA into the cell during electroporation. However, there may be other mechanisms involved, since there are many steps of gene electrotransfer on which Mg^{2+} ions can have an effect on.

Acknowledgements

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Poglavje 2

Vpliv magnezijevih ionov na različne korake genske elektrotransfekcije

The influence of Mg ions on different steps of gene electrotransfer

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V pripravi za objavo

POVZETEK

Genska elektrotransfekcija je nevirusna metoda, ki z električnimi pulzi omogoča vnos DNA v žive celice. Za uspešnost metode je potrebnih več korakov in prvi korak predstavlja interakcija DNA s celično membrano. Divalentni kationi v elektroporacijskem mediju lahko vplivajo na vezavo DNA na celično membrano in s tem na učinkovitost genske elektrotransfekcije. Cilj naše raziskave je bila zato analiza vpliva Mg^{2+} ionov med elektroporacijo na formacijo kompleksa DNA s celično membrano. Za vizualizacijo kompleksa celične membrane in DNA, obarvane s fluorescenčnim barvilom TOTO-1, smo dovedli prvi vlak pulzov v prisotnosti različnih koncentracij Mg^{2+} ionov (1 mM in 50 mM). Za analizo vpliva Mg^{2+} ionov na moč vezave DNA s celično membrano smo dovedli drugi vlak pulzov z obrnjeno polariteto. Po dovajanju drugega vlaka pulzov z obrnjeno polariteto smo opazili padec fluorescence na celični membrani pri uporabi medija z 1 mM koncentracijo Mg^{2+} ionov, medtem ko tega ni bilo opaziti pri uporabi medija s 50 mM koncentracijo Mg^{2+} ionov. Ker so znotrajcelične DNaze aktivirane z Mg^{2+} ioni in tako lahko razgradijo plazmidno DNA, smo preučevali tudi vpliv DNaznega inhibitorja Zn na učinkovitost genske elektrotransfekcije pri različnih koncentracijah Mg^{2+} ionov. Pri dodajanju $ZnSO_4$ takoj po dovajanju električnih pulzov nismo opazili izboljšanja učinkovitosti genske elektrotransfekcije. Zato sklepamo, da pri višjih koncentracijah Mg^{2+} ionov pride do tako močne vezave DNA z membrano, da ta ne more prehajati čez celično membrano in posledično pride do znižanja učinkovitosti genske elektrotransfekcije.

Ključne besede: Mg ioni, elektroporacija, interakcija DNA-celična membrana, genska elektrotransfekcija, TOTO-1, inhibitorji Dnaze.

ABSTRACT

Gene electrotransfer is nonviral method used to transfer genes into living cells and it is known to be a multi-step process. The first step is the interaction of DNA with the cell membrane. Divalent cations in the electroporation media can influence the anchoring of DNA to the membrane and by that gene electrotransfer efficiency. Therefore the aim of our study was to analyze the effect of Mg^{2+} ions on formation of DNA-membrane complex during electroporation. First train of pulses was applied in presence of different Mg^{2+} concentration (1 mM and 50 mM) to visualize complex between TOTO-1 labeled DNA and membrane. Afterwards second train of pulses with reversed polarity was applied to analyze the role of Mg^{2+} ions on the binding strength between DNA and cell membrane during electroporation. We observed that after applying reversed polarity pulses, fluorescence decrease appeared at the cell membrane in 1 mM Mg media compared to 50 mM Mg media. Because intracellular DNases are activated with Mg^{2+} ions and can decompose plasmid DNA, we also studied if the efficiency of gene electrotransfer can be improved by adding DNase inhibitor Zn immediately after the pulse application. Because we did not observe any improvement of gene electrotransfer efficiency by adding $ZnSO_4$, we suggest that Mg^{2+} ions at higher concentrations bind DNA at the cell membrane with such intensity, that DNA cannot cross the membrane during electroporation and by that influence efficiency of gene electrotransfer.

Keywords: Mg ions, electroporation, DNA-cell membrane interaction, gene electrotransfer, TOTO-1, DNase inhibitors.

1 INTRODUCTION

The electroporation is a process where sufficiently high electric field causes transient permeability of the cell membrane (1, 2). Consequently different kinds of small and large molecules can be introduced into the cytoplasm, that otherwise could not enter through cell membrane. Because of its easy application, safety and efficiency, electroporation showed great potential for use in biomedical or industry applications, such as electrofusion (3, 4), electrochemotherapy (5 - 8), irreversible electroporation (9), DNA vaccination (10), microbial non-thermal inactivation in food (11) and for gene transfection (12, 13).

A variety of methods was described to transfect genes into cells and tissues. To date viral vectors have been the most effective in transfecting genes into living cells. However, viral transfection can have serious limitations in terms of pathogenicity. (14). Therefore the need for developing a non-viral transfection method emerged, such as gene electrotransfer. Gene electrotransfer is a non-viral method that enables delivery of plasmid DNA into the cell by applying electric pulses. It was first described in early 80's (12, 15) and was since then used to introduce DNA into prokaryotic and eukaryotic cells *in vitro* and *in vivo*.

A number of steps are involved in successful gene electrotransfer: (i) formation of a complex between DNA and cell membrane, (ii) translocation of DNA across the permeabilized membrane, (iii) transfer of DNA from cytoplasm into nucleus and (iv) gene expression (16, 17). Several parameters can have influence on each of these steps (18 - 23), especially Mg^{2+} ions can influence the first step needed for successful gene electrotransfer. Namely, they can act as a bridge between negatively charged DNA and negatively charged exterior of the cell (24) and by that can influence the efficiency of gene electrotransfer. To date, only few studies have experimentally investigated the effect of Mg^{2+} ion concentration in electroporation media on gene electrotransfer (12, 25, 26). Wong et al. first showed that Mg^{2+} ions facilitate the adsorption of DNA to the cell surface (15). His study was continued by Neumann et al. where they observed that although the presence of Mg^{2+} ions increases the amount of DNA bound to the cell surface, these ions in fact reduces gene electrotransfer efficiency (12), while in study of

Xie et al. gene electrotransfer efficiency was increased (25). Zhao et al. in *in vivo* study suggested that divalent cations limit DNA entrance into the cell during electroporation due to changed stability and physical properties of nucleic acid (21).

Since plasmid DNA has to transfer through the cell cytoplasm and into the nucleus in order for gene expression to occur, also intracellular factors which could affect this process are important. It was shown, that intracellular nucleases (DNases) can decompose plasmid DNA (27) and that Mg^{2+} ions are necessary for enzymatic activity of DNases (28). In order to improve gene electrotransfer efficiency, DNase inhibitors were used (29). In this study they showed, that when applying Zn^{2+} DNase inhibitor after electric pulse application, gene electrotransfer efficiency increased.

Nevertheless, the molecular events involved in formation of a complex between cell membrane and DNA, its translocation across the membrane and through cell cytoplasm are still poorly understood, and require further investigation.

In this study we focused on the analysis of the effect of Mg^{2+} ions on formation of DNA-membrane complex during electroporation and their role on binding strength between DNA and cell membrane. Also the role of Mg^{2+} ions on DNase activity and as a consequence on gene electrotransfer efficiency was explored.

2 MATERIALS & METHODS

2.1. Cell culture and electroporation media

Chinese hamster ovary cells (CHO-K1) were grown in Ham's tissue culture medium for mammalian cells with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) at 37° C in a humidified 5% CO_2 atmosphere in the incubator (Kambič, Slovenia).

For our study we used electroporation media (10 mM KH_2PO_4/K_2HPO_4, pH = 7.2) with different concentration of Mg^{2+} ions (1 mM and 50 mM). We used different concentration of sucrose in electroporation media to maintain isoosmolar surrounding of

the cell. As it was shown by Golzio et al. (30), pulsing the cells in media with different sucrose concentration did not dramatically change transfection efficiency.

2.2. Plasmid DNA

Plasmid pEGFP-N_1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in DH5α strain of *Escherichia coli* and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

2.3. DNA staining and visualization of DNA-cell membrane interaction

To visualize DNA interaction with cell membrane TOTO-1 nucleic acid stain (Molecular Probes- Invitrogen, Carlsbad, California, USA) was used which was already shown to enable direct visualization of DNA with the cell membrane (31). The plasmid pEGFP-N_1 was labeled with 2.3 x 10^{-4} M TOTO-1 DNA intercalating dye for 60 min on ice. Plasmid concentration was 1 μg/μl, which yields an average base pair to dye ratio of 5. Cells were plated as a monolayer culture in Labtech chamber for 1 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in the incubator at cell density of ρ = 1 x 10^5 cells/ml. After 1 h culture medium was removed and replaced with electroporation media with different concentration of Mg^{2+} (1 mM and 50 mM). Immediately before electric pulse application, labeled plasmid was added to cells in concentration 10 μg/ml. Samples were then exposed to electric pulses using Jouan GHT 1287 electroporator (Jouan, St. Herblain, France). An oscilloscope Wave surfer^{TM} 422 (Le croy, Chestnut Ridge, New York, USA) was used to monitor pulse shape. First train of eight pulses with duration of 5 ms with electric field 0.7 kV/cm and repetition frequency 1 Hz was applied.

After observing interaction of DNA with the cell membrane 1 min after first pulsation, a second train of identical pulses of the opposite polarity was applied (8x5 ms, 0.7 kV/cm, 1 Hz) approximately 2 min after first pulsation. The effect of reversed

pulse polarity on the life-time of DNA-membrane complex formation was already shown by Faurie et al. (32). The distance between a pair of two wire stainless steel parallel electrodes was d = 2 mm.

To monitor the interaction of DNA with the cell membrane, fluorescent microscopy (Zeiss 200, Axiovert, Germany) was used with 100x oil immersion objective. The images (see Fig. 1) were recorded and TOTO fluorescence intensity profiles were measured and analyzed using imaging system (MetaMorph imaging system, Visitron, Germany).

2.4. Effect of DNase inhibitor on gene electrotransfer efficiency

Electroporation was performed on 24 hours old cell culture in electroporation media with different concentration of Mg^{2+} (1 mM and 50 mM).

Cell suspension was prepared by 0.25% trypsin/EDTA solution (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), centrifuged for 5 min at 1000 rpm (180 x g) at 4°C (Sigma, Germany) and resuspended in different electroporative media to a cell density of ρ = 2.5 x 10^6 cells/ml. Plasmid DNA was added to cell suspension in concentration 40 μg/ml. For electroporation, cuvettes with built in aluminium electrodes were used (Eppendorf, Hamburg, Germany). The volume of the cells placed in cuvette was 200 μl. After 2-3 min incubation of DNA with CHO cells at room temperature (22 °C), samples were exposed to square wave electric pulses using Cliniporator™ pulse generator (IGEA s.r.l., Carpi, Modena, Italy) to deliver plasmid DNA into the cells. A train of four rectangular pulses with duration of 200 μs and repetition frequency 1 Hz was applied. The applied voltage was 400 V, which resulted in 1.0 kV/cm E. Applied electric field is defined by

$$E = U(appl)/d, \quad (1)$$

where U(appl) denotes applied voltage and d electrode distance (d = 4 mm).

After exposing cells to electric pulses, fetal calf serum (FCS-Sigma, USA) was added (25% of sample volume) to preserve cell viability. Immediately after pulses also 10 µl of 80 µM $ZnSO_4$ (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was added to inhibit intracellular DNases, as was already shown by others (29).

Cells were then incubated for 5 min at 37°C to allow cell membrane resealing and then grown for 24 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in the incubator. After 24 h cells were trypsinized, centrifuged for 5 min at 1000 rpm (180 x g) at 4°C and resuspended in phosphate buffered saline (PBS) to a cell density of $\rho = 1 \times 10^6$ cells/ml. The percentage of GFP-expressing cells was determined using a flow cytometer (Coulter EPICS Altra flow cytometer, Beckman Coulter Electronics, Brea, CA), equipped with a laser emitting at 509 nm and 9 000 events were recorded.

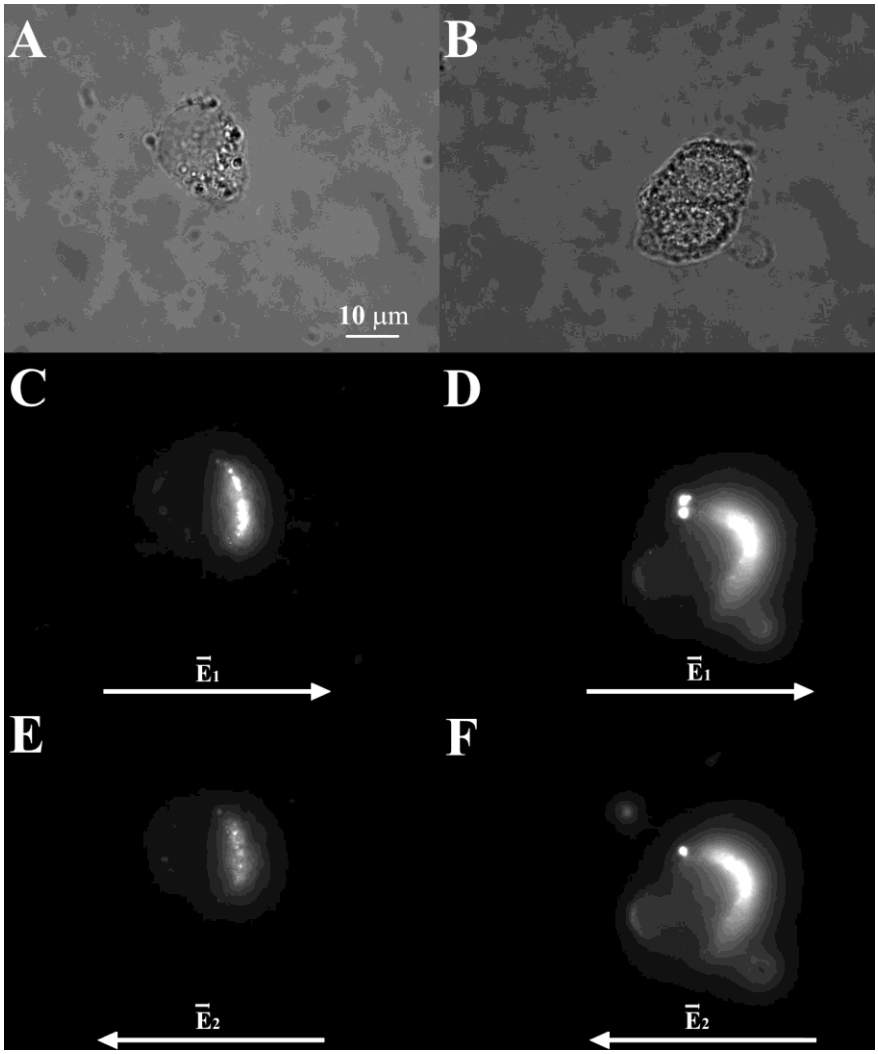
3 RESULTS

3.1. Visualization of DNA-cell membrane interaction

To go further into the mechanism of gene electrotransfer in order to understand the role of Mg^{2+} ions, direct visualization of DNA interaction with cell membrane was performed by using fluorescent plasmid DNA. For this purpose TOTO-1 labeled plasmid DNA and electroporation media with different concentration of Mg^{2+} ions (1 mM and 50 mM) was used to analyze the influence of these ions on DNA-membrane complex during electroporation (Fig. 1).

When using 1 mM and 50 mM concentration of Mg^{2+} ions, DNA interacted with cell membrane facing the cathode when the first train of pulses (8x5 ms, 0.7 kV/cm, 1 Hz) was applied.

After exposing cells to the second train of pulses of the opposite polarity (8x5 ms, 0.7 kV/cm, 1 Hz), the fluorescence decrease appeared at the membrane in media with 1 mM concentration of Mg^{2+} compared to media with 50 mM concentration of Mg^{2+}, where reversed polarity pulses did not affect the fluorescence at the cell membrane (Fig. 1).



1 mM **50 mM**

Fig. 1: Fluorescence microscopy observation of DNA-membrane interaction when using different Mg^{2+} ion concentrations. Plated cells were incubated in presence of TOTO-1 labeled DNA (pEGFP-N_1) in electroporation media with different Mg^{2+} ion concentration (1 mM and 50 mM). Concentration of labeled DNA in electroporation media was 10 μg/ml. Immediately after labeled plasmid was added to cells the first train of pulses (8x5 ms, 0.7 kV/cm, 1 Hz) was applied (C and D). The second train of pulses-pulses of the opposite polarity (8x5 ms, 0.7 kV/cm, 1 Hz) was applied 2 min after first pulsation (E and F). Phase contrast images of treated cells in (A) 1 mM and (B) 50 mM Mg electroporation media. Fluorescence images of treated cells in (C and E) 1 mM and (D and F) 50 mM Mg electroporation media. Images were acquired approximately 1 min after each pulsation. The white arrows indicate field direction of the first (E_1) and the second (E_2) train of pulses applied. The same cells (A, C, E and B, D, F, respectively) were always observed after first and second trains of pulses were applied.

In Fig. 2, TOTO fluorescence intensity profiles were measured along the membrane of the same cell after the first and the second trains of pulses were applied. The fluorescence level was not found to be homogeneous in the membrane as shown by the peaks of fluorescence (Fig. 2). In both media (1 mM and 50 mM) the increase in fluorescence caused by the first pulses varied at the cathode side from 4.5- to 11-fold compared to the anode side. This increase in fluorescence can be explained by the accumulation of the labeled DNA at the cell membrane surface, which was permeabilized and it was only present at the membrane level. After pulses of the opposite polarity were applied, approximately 1.5-fold decrease in fluorescence intensity in 1 mM Mg media was observed at the cathode side. None or very small decrease in fluorescence intensity in 50 mM Mg media was detected.

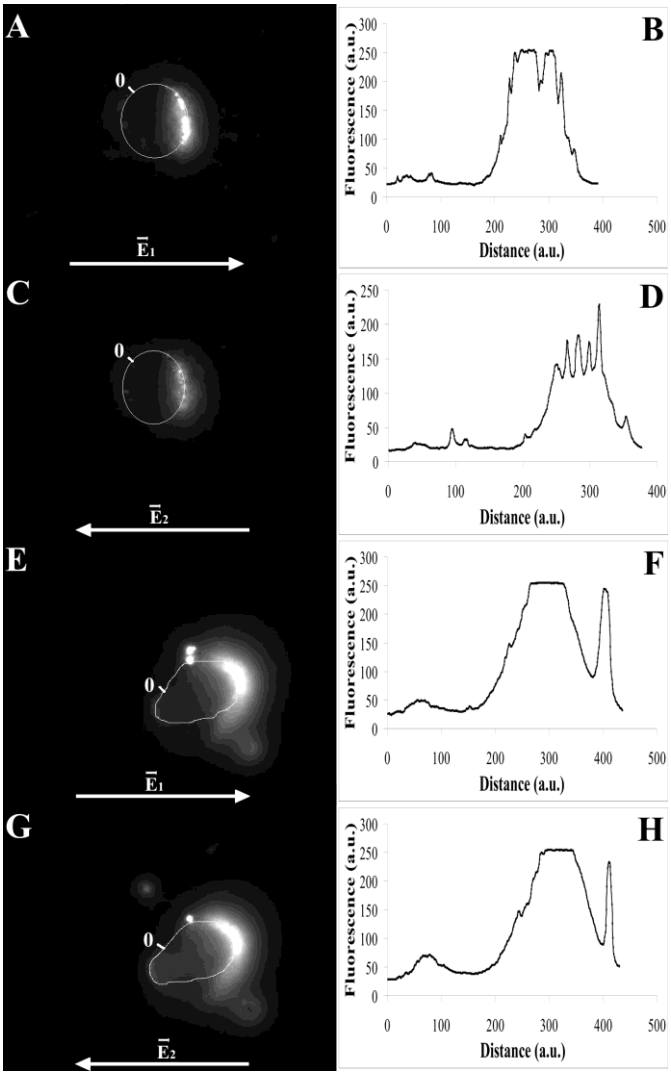


Fig. 2: Fluorescence intensity profiles at the membrane level. Plated cells were incubated in presence of TOTO-1 labeled DNA (pEGFP-N_1) in electroporation media with different Mg^{2+} ion concentration (1 mM and 50 mM). Immediately after labeled plasmid was added to cells the first train of pulses (8x5 ms, 0.7 kV/cm, 1 Hz) was applied (A and E). The second train of pulses- pulses of the opposite polarity (8x5 ms, 0.7 kV/cm, 1 Hz) was applied 2 min after first pulsation (C and G). Fluorescence images of treated cells in (A and C) 1 mM and (E and G) 50 mM Mg electroporation media. Images were acquired approximately 1 min after each pulsation. On these images the membrane was drawn in white. Fluorescence intensity profiles of the permeabilized areas (B, D, F and H) were determined by quantifying the intensity values of the fluorescence at the membrane level along the white line drawn in A, C, E and G. The white arrows indicate field direction of the first (E_1) and the second (E_2) train of pulses applied. The same cells (A, C and E, G, respectively) were always observed after first and second trains of pulses were applied.

3.2. Effect of DNase inhibitor on gene electrotransfer efficiency

To test the effect of Mg^{2+} ions on nuclease inhibitors and by that on gene electrotransfer efficiency, 40 μg/ml of pGFP-N_1 was added into electroporation media. Cells were electroporated with 40 μg/ml pGFP-N_1 with 4x200 μs pulses at 1.0 kV/cm, 1 Hz, in the presence of $ZnSO_4$ in electroporation media with 1 mM and 50 mM concentration of Mg^{2+} ions. It was already shown, that DNase inhibitor $ZnSO_4$ has a profound effect on gene electrotransfer efficiency, if it is added immediately after pulse application (29). Therefore we used the same protocol.

We did not observe any improvement of gene electrotransfer efficiency by adding DNase inhibitor $ZnSO_4$ in both electroporation media (1 mM and 50 mM Mg media) (Fig. 3). The percentage of transfected cells was approximately the same as if no $ZnSO_4$ was added.

Adding 80 mM concentration of $ZnSO_4$ did not affect cell viability (data not shown), but treatment of cells with higher concentration of $ZnSO_4$ (up to 2 M) resulted in lower or no cell viability.

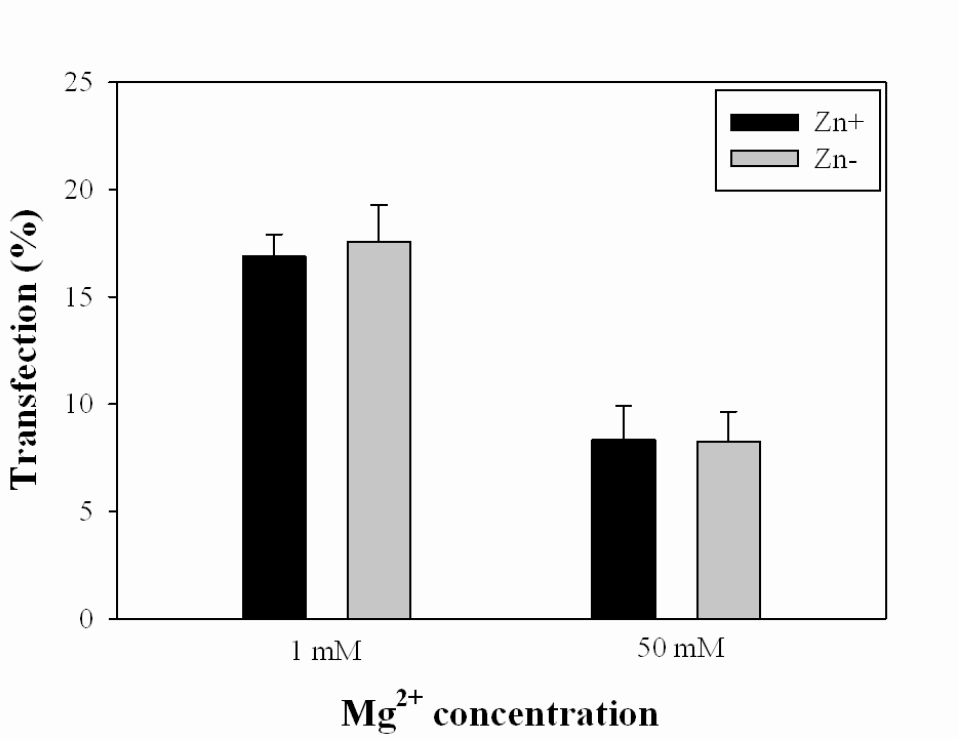


Fig.3: Effect of DNase inhibitor ($ZnSO_4$) on gene electrotransfer efficiency as a function of different Mg^{2+} concentration in electroporation media (1 mM and 50 mM) is shown: (black histogram) when $ZnSO_4$ was added, or (grey histogram) no $ZnSO_4$ was added. 4 x 200 μs pulses with electric field strength of 1.0 kV/cm and repetition frequency 1 Hz were used at room temperature (T = 22 °C). Cell density ρ = 2.5 x 10^6 cells/ml. Values represents means ± standard deviation.

4 DISCUSSION & CONCLUSIONS

Gene electrotransfer is a method that enables delivery of plasmid DNA into the cell by means of electric pulses. One of the main concerns in gene electrotransfer is still its efficiency. There are few reports that indicated the effect of divalent cations on DNA adsorption to the cell membrane during pulse application, which could influence the efficiency of gene electrotransfer (12, 15, 21, 24, 25). It was also shown, that higher concentration of Mg^{2+} ions leads to lower gene electrotransfer efficiency (12, 26). Therefore the aim of our study was to expand our previous research (26) in order to

investigate the effect of Mg^{2+} ions on strength of adsorption of negatively charged DNA onto negatively charged cell membrane during electroporation and to see, if Mg^{2+} ions increase activity of DNAse enzyme, which also affect gene electrotransfer efficiency.

To analyze the effect of Mg^{2+} concentration on the formation of a complex between DNA and cell membrane, visualization of DNA interaction with the cell membrane using TOTO-1 nucleic acid stain was performed in media with 1 mM and 50 mM Mg. After applying electric pulses (8x5 ms, 0.7 kV/cm, 1 Hz) localized spots of stained DNA was observed on the cell membrane facing the cathode for both Mg media (see Fig. 1), similarly as in Golzio et al. (31). Afterwards cells were exposed to electric pulses of the opposite polarity (8x5 ms, 0.7 kV/cm, 1 Hz) to analyze the effect of Mg^{2+} ions on stability of the DNA-membrane complex. We observed that after applying pulses of the opposite polarity the fluorescence decrease on cell membrane was observed when 1 mM Mg media was used (see Fig. 1C and 1E). Therefore the complex between DNA and cell membrane was disrupted. None or very small fluorescence decrease on cell membrane was observed when 50 mM Mg media was used (see Fig. 1D and 1F).

Nucleases are responsible for plasmid degradation before their entry into the nucleus (27). It was shown that for some conditions divalent ions (mostly Mg^{2+}) increase activity of DNase enzyme (28), which as a result can decrease the gene electrotransfer efficiency. To test if the decrease in gene electrotransfer efficiency with electroporation media with high concentration of Mg^{2+} ions (26) is a cause of increased DNase activity, we added DNase inhibitor $ZnSO_4$ immediately after electroporation. Although in previous study they observed the increase in gene electrotransfer efficiency when $ZnSO_4$ was added after pulse application (29), we did not observe such effect (see Fig. 3). We also added higher concentration of $ZnSO_4$ (up to 2 M) in order to exclude the idea, that we used too low Zn^{2+} concentration (80 μM) to have any effect on inhibition of DNase enzymes. For those conditions we observed almost no cell viability (data not shown). No improvement in cell viability was observed when cells were centrifugated after receiving the electric pulses in order to remove any potentially toxic extracellular $ZnSO_4$. It is possible, that more potent DNase inhibitors, such as aurintricarboxylic acid or its combination with Zn^{2+} should be used for our conditions. It is also important to stress, that Zn^{2+} ions (as divalent cations) do not facilitate the

interaction of DNA with the cell membrane, since they are added after electric pulse application, when first step is already ended. After electric pulses are applied, resealing of the membrane is occurring and translocation of the plasmid to the cytoplasm already takes place (16).

By applying pulses with reversed polarity, we have shown that disruption of interaction between DNA and cell membrane when using 50 mM Mg media does not take place.

Our results therefore suggest that Mg^{2+} ions at higher concentrations bind DNA at the cell membrane at such intensity, that second step needed for successful gene electrotransfer efficiency (translocation of DNA across the cell membrane) is hindered during electroporation.

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Poglavje 3

Uporaba kolagenskega gela kot 3-D model in vitro za študijo elektropermeabilizacije in genske elektrotransfekcije

Use of collagen gel as a three-dimensional in vitro model to study electropermeabilization and gene electrotransfer

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POVZETEK

Genska elektrotransfekcija je obetavna nevirusna metoda, ki omogoča vnos DNA v celice z dovajanjem električnih pulzov. Kljub temu, da so bile narejene številne raziskave *in vivo* ter *in vitro*, sami mehanizmi genske elektrotransfekcije še niso poznani. Glavni problem uspešnosti genske elektrotransfekcije *in vivo* predstavlja slaba mobilnost DNA v tkivu. Ker so celice vpete v ekstracelularno ogrodje in se zato obnašajo drugače kot celice v standardnih pogojih *in vitro*, smo razvili tridimenzionalni (3-D) model *in vitro* CHO celic, gojenih v kolagenskem gelu. Naš 3-D model tako predstavlja *ex vivo* model tkiva za študijo elektropermeabilizacije in različnih parametrov genske elektrotransfekcije. Za detekcijo elektropermeabilizacije CHO celic, gojenih v kolagenskem gelu, smo uporabili propidijev jodid. Sledila je analiza vpliva različnih koncentracij plazmidne DNA in dolžine pulza na učinkovitost genske elektrotransfekcije. Naši rezultati so pokazali, da je bila genska elektrotransfekcija kljub uspešni elektropermeabilizaciji precej nizka. Učinkovitost genske elektrotransfekcije v 3-D modelu *in vitro* je bila odvisna od koncentracije plazmidne DNA in dolžine pulza, podobno kot v študijah *in vivo*, kjer so dokazali, da so daljši (milisekundni) pulzi bolj optimalni kot krajši (mikrosekundni) pulzi. Naši rezultati prikazujejo, da je 3-D model *in vitro* bolj primerljiv s stanjem *in vivo* kot klasična 2-D celična kultura in kot tak omogoča študijo mehanizmov genske elektrotransfekcije v *in vivo* podobnem okolju.

Ključne besede: 3-D model *in vitro*, kolagenski gel, genska elektrotransfekcija, GFP, elektropermeabilizacija, propidijev jodid, CHO celica.

Izjava: Podpisana Saša Haberl izjavljam, da članka, *Use of collagen gel as a three-dimensional in vitro model to study electropermeabilization and gene electrotransfer,* objavljenega v reviji Journal of Membrane Biology 2010 (236): 87−95, nihče od soavtorjev ni uporabil za svojo doktorsko disertacijo.

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Use of Collagen Gel as a Three-Dimensional In Vitro Model to Study Electropermeabilization and Gene Electrotransfer

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Abstract Gene electrotransfer is a promising nonviral method that enables transfer of plasmid DNA into cells with electric pulses. Although many in vitro and in vivo studies have been performed, the question of the implied gene electrotransfer mechanisms is largely open. The main obstacle toward efficient gene electrotransfer in vivo is relatively poor mobility of DNA in tissues. Since cells are mechanically coupled to their extracellular environment and act differently compared to standard in vitro conditions, we developed a three-dimensional (3-D) in vitro model of CHO cells embedded in collagen gel as an ex vivo model of tissue to study electropermeabilization and different parameters of gene electrotransfer. For this purpose, we first used propidium iodide to detect electropermeabilization of CHO cells embedded in collagen gel. Then, we analyzed the influence of different concentrations of plasmid DNA and pulse duration on gene electrotransfer efficiency. Our results revealed that even if cells in collagen gel can be efficiently electropermeabilized, gene expression is significantly lower. Gene electrotransfer efficiency in our 3-D in vitro model had similar dependence on concentration of plasmid DNA and pulse duration comparable to in vivo studies, where longer (millisecond) pulses were shown to be more optimal compared to shorter (microsecond) pulses. The presented results demonstrate that our 3-D in vitro model resembles the in vivo situation more closely than conventional 2-D cell cultures and, thus, provides an environment closer to in vivo conditions to study mechanisms of gene electrotransfer.

Keywords 3-D in vitro model · Collagen gel · Gene electrotransfer · GFP · Electropermeabilization · Propidium iodide · CHO cell

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Introduction

A variety of biochemical methods have been developed to transfer genes into cells, but many of them have either low efficiency or potential side effects (Curiel et al. 1991; Wagner et al. 1992; Cotten and Wagner 1993; Simoes et al. 1998; Marshall 1999; Kikuchi et al. 1999; Hacein-Bey-Abina et al. 2002). In the 1970s a physical method named "electropermeabilization," or "electroporation," was described to introduce molecules into cells, where a temporary increase in membrane permeability was achieved by electric pulses (Neumann and Rosenheck 1972). The first biomedical application of electropermeabilization was developed in the late 1980 s for introducing poorly permeant anticancer drugs into cutaneous and subcutaneous tumor nodules (Mir et al. 1991, 1998; Miklavcic et al. 1998; Sersa et al. 2000; Sersa 2006; Byrne and Thompson 2006). It was also shown that electroporation of cells in physical contact induces cell fusion, a process known as "electrofusion" (Vienken and Zimmermann 1985; Usaj et al. 2010). In the 1980s successful transfection of a gene into eukaryotic cells by applying electric pulses was achieved (Neumann et al. 1982; Potter 1988). Gene electrotransfer has since, due to its ease of application and efficiency, become a routine method for introducing foreign genes into bacterial (Drury 1996), yeast (Simon 1993), plant (Terzaghi and Cashmore 1997), and animal

(Andreason and Evans 1989; Rols and Teissie 1998) cells in vitro and into different tissues, including muscle (Aihara and Miyazaki 1998; Mir et al. 1999), tumors (Nishi et al. 1996; Rols et al. 1998), liver (Heller et al. 1996), and skin (Titomirov et al. 1991) in vivo. Nevertheless, mechanisms involved in gene electrotransfer in vitro or in vivo remain largely unknown.

The main obstacle for in vivo gene electrotransfer remains its efficiency since in tissues the diffusion of large molecules is impaired (Rols et al. 1998). Namely, extracellular matrix is thought to be one of the major barriers for successful gene electrotransfer in vivo (Zaharoff et al. 2002; Zaharoff and Yuan 2004; Cemazar et al. 2006).

Up to now many researchers have experimentally investigated various electroporation protocols to optimally deliver plasmid DNA into different tissues in vivo (Rols et al. 1998; Bettan et al. 2000; Cemazar et al. 2002; Andre et al. 2008; Tevz et al. 2008).

However, in vivo studies require large numbers of killed animals, and there are many factors (properties of tissue, such as differences in tissue organization, presence or absence of necrosis, overall tissue conductivity, the ability of cells to express transfected genes, cell density, and cell size) that influence gene electrotransfer efficiency (Somiari et al. 2000; Bettan et al. 2000). For this reason, the development of a reproducible three-dimensional (3-D) in vitro model of tissue is important since it would enable in vitro studies of gene electrotransfer, while classical in vitro experiments use plated monolayer cells which dramatically differ from cells in a 3-D environment.

Currently, different 3-D in vitro models of cell cultures are being employed in many areas of biomedical research, such as studying cell growth and mobility (Harkin and Hay 1996; Cukierman et al. 2001; Barralet et al. 2005; Hindie et al. 2006), tissue engineering (Chevallay and Herbage 2000) and cancer research (Kim et al. 2004; Lee et al. 2007). Also, studying cell behavior and differentiation in a simple 3-D in vitro model such as 3-D cell pellets has been reported (Ong et al. 2006; Bernstein et al. 2009). In addition 3-D spheroid models were used as models of tumors for analyzing transport of small molecules (Canatella et al. 2004; Wasungu et al. 2009) and for gene electrotransfer (Wasungu et al. 2009). However, up to now there has been no analysis of gene electrotransfer in a 3-D in vitro model made of collagen gel.

Therefore, the aim of our study was to develop a 3-D model which would enable studies of gene electrotransfer in an environment closer to in vivo conditions. We used CHO cells embedded in 3-D collagen gels to study electropermeabilization with uptake of propidium iodide (PI) and the effect of different plasmid concentrations and pulse durations on gene electrotransfer efficiency.

Materials and Methods

Preparation of Collagen Gel with Embedded Cells

Type I collagen from rat tail was obtained from Sigma-Aldrich (Deisenhofen, Germany) as a powder. Collagen solution was prepared on ice with diluted acetic acid (28.5 ml glacial acetic acid/l) to achieve a collagen concentration of 4.0 mg/ml and stored at 4°C.

After 24 h, collagen mixture was prepared by mixing 2.3 parts chilled collagen solution with 0.5 part Ham tissue culture medium for mammalian cells with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 0.5 part 1× phosphate-buffered saline (PBS, pH 7.4). The pH of the mixture was adjusted to 7.2–7.6 with 0.1 M NaOH. To prevent gelation, the temperature of the mixture was maintained at 2–8°C.

Chinese hamster ovary cells (CHO-K1) were prepared as a cell suspension by 0.25% trypsin/EDTA solution (Sigma-Aldrich) and centrifuged for 5 min at 1,000 rpm (180×g) at 4°C (Sigma-Aldrich). The cell pellet was resuspended with a liquid collagen mixture to a cell density of $\rho = 5.6 \times 10^5$ cells/ml. Collagen mixture (180 µl) with cells was pipetted into each space of a multiwell dish and stored for 1 h at 37°C in a humidified 5% CO_2 atmosphere in an incubator (Kambič, Semič, Slovenia). After raising the temperature to 37°C, collagen polymerized and formed a gel with embedded cells. Warm Ham culture medium for mammalian cells was gently added on top of the cells embedded in a gel as for normal culture. The plate was returned to the incubator and stored for 24 h at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid DNA

Plasmid pEGFP-N1 (Clontech, Mountain View, CA) encoding green fluorescent protein (GFP) was amplified in DH5α strain of *Escherichia coli* and isolated with the HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). The plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

Electropermeabilization

Standard electroporative medium (200 µl, pH 7.4; 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$ and 250 mM sucrose) with 6 µl of 0.15 mM PI was added on top of CHO cells embedded in collagen gel. PI is a small molecule which enters a cell if the membrane of the cell is permeabilized (Ganeva et al. 1995). After 3-min incubation at room temperature (22°C), different pulsing protocols were used to monitor penetration of PI.

First, eight pulses lasting 5 ms at a frequency of 1 Hz were applied, with different electric field strengths

(0.2–1.2 kV/cm). Then, different pulse durations (8 × 200 μs, 8 × 1 ms, 8 × 5 ms, 8 × 10 ms) were used to deliver PI into the cells. In this experiment, an electric field strength of 0.8 kV/cm (applied voltage $U = 320$ V) and a repetition frequency of 1 Hz were used.

In all experiments, a pair of two-plate stainless-steel parallel electrodes with interelectrode distance $d = 4$ mm was used, which provided a homogenous electric field between the electrodes, $E = U/d$, where U is the applied voltage. Electrodes were dipped vertically into collagen gel, where cells were embedded and all cells in the 3-D collagen gel between the electrodes were exposed to the homogenous electric field.

For pulsing we used the GHT 1287 electroporator (Jouan, St. Herblain, France). An oscilloscope Wave Surfer^{TM} 422 (Le Croy, Chestnut Ridge, NY) monitored pulse shape. No electric pulses were applied to cells in the control sample.

PI transport into cells was monitored by observing the fluorescence of PI, which occurred when PI entered electropermeabilized cells. Fluorescence was detected by fluorescent microscopy (Axiovert 200; Zeiss, Gottingen, Germany) with excitation light at 530 nm generated with a monochromator system (PolyChrome IV; Visitron, Puchheim, Germany), and emission was detected at 617 nm. Images (see Fig. 2) were recorded using the MetaMorph imaging system (Visitron).

Gene Electrotransfer

Our study was divided into two sets of experiments. In the first part, the optimal concentration of added plasmid DNA was determined for our experimental conditions. In the second part, different pulse durations were tested for optimal concentration of plasmid DNA, as previously established. Electroporation was performed on 24-h-old cell culture with standard electroporative medium (pH 7.4; 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$ and 250 mM sucrose).

On the day of the experiment, culture medium was removed and cells embedded in collagen gel were incubated with given concentrations of plasmid DNA that codes for GFP in electroporative media for 30 min at room temperature (22°C).

To determine the optimal concentration of plasmid DNA, cells embedded in collagen gel were incubated with different concentrations of plasmid DNA in electroporative medium (10, 60, 90, 125 μg/ml). The volume of added electroporative medium with DNA was 200 μl. A train of eight square pulses with duration of 5 ms and repetition frequency of 1 Hz was applied to deliver DNA into the cells. The amplitude of electric pulses applied was 320 V, which resulted in a homogeneous electric field between the electrodes of 0.8 kV/cm. No electric pulses were applied to cells in the control sample.

In the second part, the effect of different pulse durations on gene electrotransfer efficiency and cell viability was tested. Cells embedded in collagen gel were incubated with the optimal concentration of plasmid DNA, which was shown to be 90 μg/ml. Electric pulses of different durations were used to deliver DNA into the cells: 8 × 200 μs, 8 × 1 ms, 8 × 5 ms and 8 × 10 ms. Electric field strength was 0.8 kV/cm (applied voltage $U = 320$ V), with repetition frequency of 1 Hz for all pulsing protocols. For pulsing, the Jouan GHT 1287 electroporator was used. The distance between a pair of two-plate stainless-steel parallel electrodes was $d = 4$ mm. Electrodes were dipped vertically into collagen gel, where cells were embedded and all cells in the 3-D collagen gel between the electrodes were exposed to the homogenous electric field.

After exposing cells to electric pulses 70 μl of FCS (Sigma, St. Louis, MO) was added (35% of sample volume) to preserve cell viability. Cells were then incubated for 15 min at 37°C to allow cell membrane resealing and grown for 24 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in the incubator.

Gene electrotransfer efficiency was determined by fluorescent microscopy (Axiovert 200) with excitation light at 445 nm generated with a monochromator system (PolyChrome IV), and emission was detected at 488 nm. Images (see Fig. 1) were recorded using the MetaMorph imaging system. At least 10 fluorescence images were acquired in the area between the electrodes at ×10 objective magnification per parameter. Cells were counted manually and gene electrotransfer efficiency was determined by the ratio between the number of green fluorescent cells (successfully transfected) and the total number of cells. Two or three independent experiments were performed for each parameter, and the results are presented as mean values ± standard deviation.

Cell Viability

Cell viability was determined by measuring PI uptake 24 h after applying pulses with different durations. PI enters the cell if the membrane is damaged. Culture medium was removed and 200 μl of PBS with 6 μl of 0.15 mM PI was added to cells. After 5-min incubation, cell viability was determined by fluorescent microscopy. Images were recorded using the MetaMorph imaging system.

At least five fluorescence images were acquired in the area between the electrodes at ×10 objective magnification per parameter. Cells were counted manually and cell viability was determined by the ratio between the number of dead cells (cells with incorporated PI) and the total number of cells.

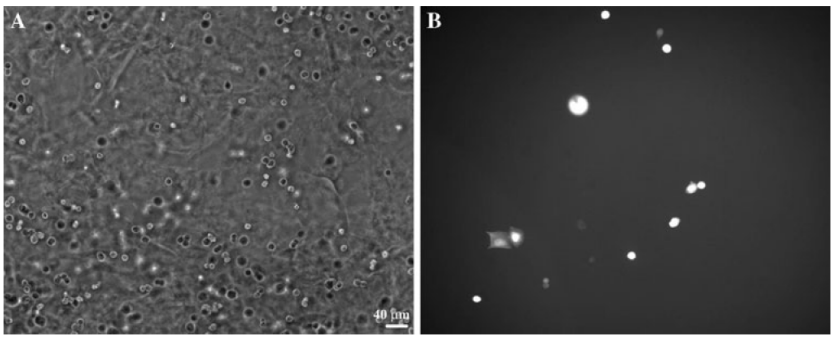

Fig. 1 Gene electrotransfer of CHO cells embedded in collagen gel 24 h after pulse application. Pulses of 8 × 10 ms ($E = 0.8$ kV/cm) were applied with repetition frequency of 1 Hz to deliver pEGFP (concentration of DNA in electroporative medium 90 μg/ml) into cells. **a** Phase-contrast image of treated cells. **b** Fluorescent image of cells expressing GFP protein (*white*). To visualize and quantify transfection, ×10 objective magnification was used

The total number of cells (for determining gene electrotransfer efficiency and cell viability) was difficult to obtain from phase-contrast imaging in 3-D. For this reason, we first determined at which pulsing parameters the entire cell population was permeabilized to PI. This we obtained for eight pulses lasting 5 ms, with an electric field strength of 1.2 kV/cm (applied voltage $U = 480$ V) and repetition frequency of 1 Hz. Therefore, after 5-min incubation with PI (described above), samples were exposed to electric pulses to permeabilize the whole cell population using the Jouan GHT 1287 electroporator. The distance between a pair of two-plate stainless-steel parallel electrodes was $d = 4$ mm. At least 10 fluorescence images were acquired in the area between the electrodes at ×10 objective magnification per parameter. Cells were counted manually.

Results

The main objective of our study was to develop a 3-D in vitro model of cells embedded in collagen gel which mimics the extracellular environment in tissues, to study the effect of different parameters on gene electrotransfer. Namely, a 3-D in vitro model would provide a more physiologically relevant approach for the analysis of gene electrotransfer than the conventional in vitro 2-D cell culture.

In the first part of the study, PI was used as a marker of efficient electropermeabilization and the electropermeabilization threshold was determined. In the second part of the study, gene electrotransfer of cells embedded in collagen gel was performed (Fig. 1). The effect of different concentrations of DNA on gene electrotransfer efficiency and the effects of pulse duration on gene electrotransfer efficiency and cell viability were analyzed.

Also, cell viability in our 3-D in vitro model was tested in control samples (samples where no electric pulses were applied), where >98% of cells survived (data not shown).

Electropermeabilization

Uptake of the impermeable dye PI was used to monitor electropermeabilization of CHO cells embedded in collagen gel and to determine the electropermeabilization threshold. Cells were subjected to eight pulses lasting 5 ms with different electric field strengths from 0.1 to 1.2 kV/cm. At 0.1 kV/cm, cells were not permeabilized (data not shown), while at 0.2 kV/cm, some cells were permeabilized (Fig. 2a). At 0.4 kV/cm approximately half of the cell population in collagen gel was permeabilized (Fig. 2b), and above 0.6 kV/cm the entire cell population was permeabilized to PI, meaning that for all cells the threshold for electropermeabilization was achieved (Fig. 2c–f).

We also tested different pulse durations (8 × 200 μs, 8 × 1 ms, 8 × 5 ms, 8 × 10 ms) to deliver PI into the cells embedded in collagen. The applied electric field strength was 0.8 kV/cm. At 8 × 200 μs, >80% of the cell population was permeabilized, while at 8 × 1, 8 × 5 and 8 × 10 ms, all cells in collagen gel were permeabilized (data not shown).

Gene Electrotransfer

To establish which plasmid DNA concentration is optimal for our protocol, we first determined the efficiency of gene electrotransfer for different plasmid concentrations. The efficiency of the transferred GFP gene was analyzed by fluorescent microscopy 24 h after electric pulse application. The electroporation protocol was the same for all concentrations of DNA: applied electric field strength 0.8 kV/cm, 8 × 5 ms pulses with 1-Hz repetition frequency.

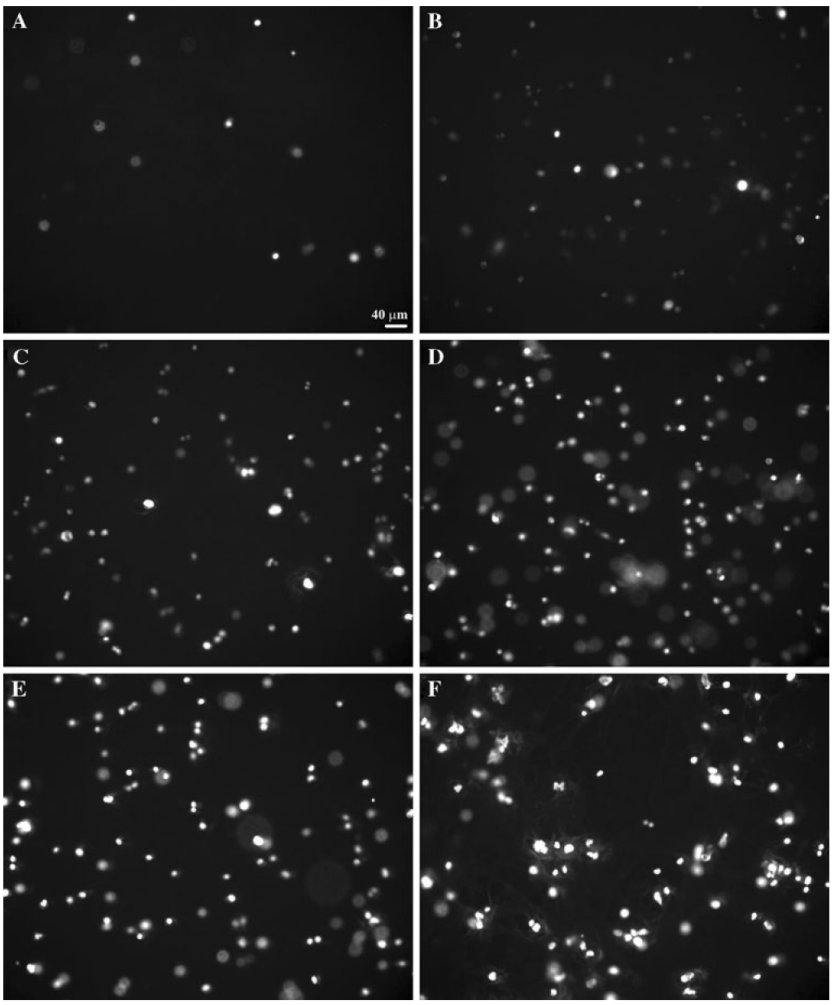

Fig. 2 Electropermeabilization of cells embedded in collagen gel. Pulses of 8 × 5 ms with repetition frequency of 1 Hz were applied to deliver PI into cells. Electric field strengths were as follows: **a** 0.2 kV/cm, **b** 0.4 kV/cm, **c** 0.6 kV/cm, **d** 0.8 kV/cm, **e** 1.0 kV/cm and **f** 1.2 kV/cm. To visualize permeabilization, ×10 objective magnification was used

As shown in Figure 3, the percentage of transfection, which represents the efficiency of gene electrotransfer, increased with increasing concentration of plasmid DNA up to 90 µg/ml, where 5.8% of cells were transfected. Increasing plasmid DNA concentration above 90 µg/ml did not result in increased gene electrotransfer efficiency. This observation correlated with a decrease in cell viability for DNA concentrations above 90 µg/ml (data not shown).

In the next part of our study we tested different pulse durations using the optimal plasmid DNA concentration, 90 µg/ml. The applied electric field of 0.8 kV/cm and repetition frequency of 1 Hz were the same for all pulse durations.

Figure 4 shows the percentage of transfection, which represents the gene electrotransfer efficiency for different pulse durations. We obtained a gradual increase in gene electrotransfer efficiency for increasing pulse durations. The highest efficiency was obtained when we applied a train of eight pulses with duration of 5 ms. Under this condition 2.5% of viable cells were transfected. With increasing pulse duration, a decrease in cell viability was observed (see Fig. 5).

Cell Viability

With increasing pulse duration, a decrease in cell viability was observed, as previously shown in vitro (Rols and Teissie 1998). Figure 5 shows the percentage of viable cells embedded in collagen gel for different pulse durations. The highest viability was observed when we applied shorter pulses (8 × 200 µs). Under this condition, 82% of cells survived. At longer pulses, the viability of cells was

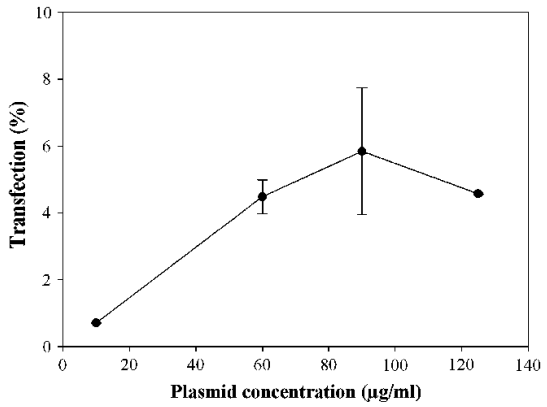

Fig. 3 Effect of different concentrations of plasmid DNA in electroporative medium on gene electrotransfer of cells embedded in collagen gel. Pulses of 8 × 5 ms (E = 0.8 kV/cm) with repetition frequency of 1 Hz were applied. The percentage of transfected cells is plotted as a function of different plasmid DNA concentrations. Each value represents the mean of two different experiments ± standard deviation

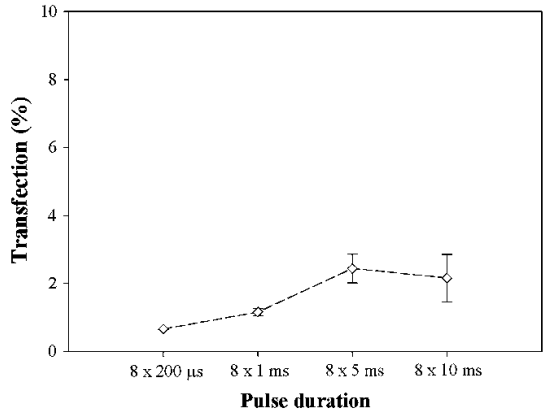

Fig. 4 Effect of different pulse durations on gene electrotransfer of cells embedded in collagen gel. Pulses of different durations with repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. The percentage of transfected cells is plotted as a function of different pulse durations. Each value represents the mean of three different experiments ± standard deviation. Plasmid concentration in electroporative medium was 90 µg/ml

significantly lower. At 8 × 10 ms, only 41% of cells survived.

Discussion and Conclusion

Transfer of DNA into the cells by membrane electroporation is an established method for gene delivery both in vitro

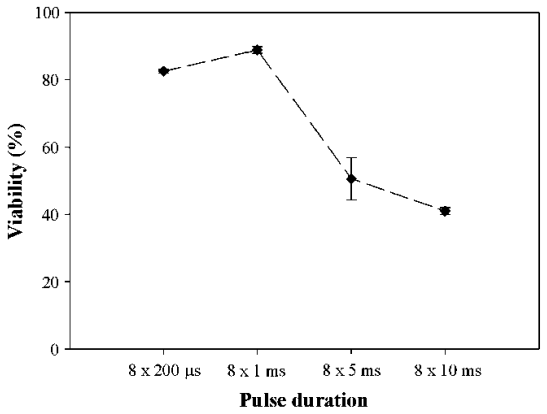

Fig. 5 Effect of different pulse durations on viability of cells embedded in collagen gel. Pulses of different durations with repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. The percentage of viable cells is plotted as a function of different pulse durations. Each value represents the mean of two to three different experiments ± standard deviation. Plasmid concentration in electroporative medium was 90 µg/ml

and in vivo. The main problem in gene electrotransfer of mammalian cells in vivo is currently its relatively low efficiency (Rols et al. 1998; Bettan et al. 2000; Cemazar et al. 2002; Tevz et al. 2008). It has been shown (Zaharoff et al. 2002; Zaharoff and Yuan 2004; Cemazar et al. 2006) that extracellular matrix presents a major obstacle for diffusion of DNA through tissue, which hinders transport of DNA in the proximity of cells, consequently leading to relatively low transfection. In order to optimize protocols and to understand the mechanisms of gene electrotransfer in vivo, it is crucial to study gene electrotransfer in a realistic 3-D in vitro model.

Therefore, the aim of our study was to develop a 3-D in vitro model of cells embedded in the extracellular environment, which would more closely mimic in vivo conditions and enable successful electropermeabilization and gene electrotransfer of cells. For efficient gene electrotransfer it is crucial that cells are exposed to E above the electropermeabilization threshold (Wolf et al. 1994). Thus, in the first part of our study, PI was used to determine the threshold for CHO cells embedded in collagen type I gel. Electropermeabilization with our electric pulses (8 × 5 ms, 1 Hz) occurred for field strengths above 0.4 kV/cm. Increasing the applied field strength resulted in an increase in electropermeabilization efficiency (see Fig. 2), as obtained in vitro and in vivo (Wolf et al. 1994; Rols and Teissie 1998; Gehl and Mir 1999; Muller et al. 2001; Sukhorukov et al. 2005).

We further analyzed electropermeabilization in our 3-D in vitro model for different pulse durations. We found that uptake of PI was efficient for all pulse durations, where slightly higher uptake was achieved when longer pulses

were used (data not shown), which is in agreement with a previous study on plated cells (Rols and Teissie 1998).

In the second part of our study, we analyzed gene electrotransfer in our 3-D collagen model. First, we determined the optimal plasmid DNA concentration for our experimental conditions. Gene electrotransfer efficiency increased with increasing concentration of plasmid DNA up to a plasmid concentration of 90 μg/ml (total amount of DNA 34 μg per sample), where approximately 5.8% of cells were transfected, which is comparable to the results obtained from studies in vivo (Cemazar et al. 2002; Andre et al. 2008). Although in this case the standard deviation was quite high, further results showed that reproducibility of the experiments was successfully achieved. A further increase in the DNA concentration resulted in a decrease of gene electrotransfer efficiency. This observation is also in agreement with a study performed in vitro, where it was shown that a higher concentration of plasmid DNA reduces gene electrotransfer efficiency due to the fact that DNA acts as a toxic substance (Wolf et al. 1994).

The electric pulse protocol is one of the most important parameters that determine gene electrotransfer efficiency. As previously demonstrated, longer pulses substantially increase gene electrotransfer efficiency (Rols and Teissie 1998). Namely, electric pulses of longer duration are supposed to contribute to the electrophoretic mobility of DNA toward and into cells (Rols and Teissie 1998; Satkauskas et al. 2002; Cemazar et al. 2006; Kanduser et al. 2009; Pavlin et al. 2010). In our study we therefore used different pulse durations in order to determine the most efficient pulse duration for our 3-D in vitro model. In all experiments, $E = 0.8$ kV/cm was used, which is above the threshold for permeabilization of the cell membrane for all pulsing protocols. The result of our experiments showed that for optimal plasmid concentrations gene electrotransfer efficiency increased with increasing duration of pulses up to 8×5 ms. This is in agreement with studies in vivo on muscle (Bureau et al. 2000; Satkauskas et al. 2002, 2005), where longer pulses distinctly increased gene electrotransfer efficiency. However, at longer pulses cell viability in our 3-D in vitro model dropped (see Fig. 5), which is in agreement with other studies, where it was suggested that the pulsing protocol should be optimized to obtain sufficient gene electrotransfer efficiency and to avoid irreversible cell damage (Rols and Teissie 1998; Mir et al. 1999).

Since the main concern for in vivo gene electrotransfer efficiency is impaired DNA diffusion through the extracellular matrix, we examined whether GFP expression was homogenous in our 3-D model. As previously noticed by Wasungu et al. (2009) in spheroids, in our 3-D model more transfected cells were detected on the top of collagen gel (data not shown). Namely, in the 3-D model and in spheroids weak DNA diffusion was detected (in our model due to collagen gel mesh and in the spheroid model due to close cell contact). Since for efficient transfection DNA has to be in close proximity to cells, high transfection in the 3-D model was obtained only in part of the gel, where there was enough DNA. The lower layers of cells could not be transfected since DNA did not diffuse enough through the collagen gel, which acts as a physical barrier.

To conclude, 2-D cell cultures do not reproduce the morphology and biochemical features that cells possess in the original tissue. As an alternative, 3-D in vitro models of cells offer the possibility to study different parameters of gene electrotransfer under conditions that more closely resemble the in vivo situation. We successfully developed such a model using collagen gel with viable embedded CHO cells, which could be used to study mechanisms of gene electrotransfer as well as to design better protocols for in vivo gene electrotransfer.

We demonstrated that gene electrotransfer efficiency in our 3-D in vitro model depends on the concentration of DNA and that the pulse duration dependence is comparable to in vivo studies (Bureau et al. 2000; Satkauskas et al. 2002, 2005; Cemazar et al. 2002; Andre et al. 2008).

Our results revealed that although small molecules, such as PI, are efficiently transferred into cells in a 3-D in vitro model, gene expression was substantially lower due to poor diffusion of plasmid DNA in collagen gel. Even though some conditions need to be optimized for our 3-D model (better cell viability, better incorporation of cells in collagen mesh), it represents an intermediate step between 2-D in vitro and animal in vivo experiments. Thus, it can be used to optimize gene delivery in vivo and reduce the number of animals used for in vivo experiments.

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Dodatek k poglavju 3

Izdelava 3-D modela *in vitro* za študijo genske elektrotransfekcije

Development of a 3-D *in vitro* model to study gene electrotransfer

1 UVOD

V bolj kompleksnih celičnih sistemih, kot so tkiva in organi, na učinkovitost genske elektrotransfekcije vplivajo struktura tkiva, interakcija celic s komponentami zunajceličnega ogrodja (proteoglikani, polisaharidi, kolagenskimi in drugimi vlakni) (1) in predvsem zmanjšana mobilnost DNA v zunajceličnem ogrodju (2 - 4). Slaba difuzija DNA v tkivu onemogoča tvorbo kompleksa DNA z membrano celice, ki je nujni pogoj za uspešno gensko elektrotransfekcijo. Tako je trenutno glavna ovira genske elektrotransfekcije *in vivo* slabša učinkovitost metode. Zato je potreben razvoj 3-D modela *in vitro*, kjer bi v celice s pomočjo elektroporacije uspešno vnašali DNA, tak model pa bi predstavljal bolj realno okolje *in vivo* kot celična kultura *in vitro*. Obenem bi model omogočal študijo optimizacije protokolov in razumevanje mehanizmov genske elektrotransfekcije brez potrebe po velikem številu žrtvovanih živali, kot je to praksa pri študijah *in vivo*. Različni 3-D modeli celičnih kultur *in vitro* se že uporabljajo pri številnih raziskavah na področju celične biologije (5 - 7). Prav tako so za analizo prehajanja učinkovin in DNA v tumorje s pomočjo električnih pulzov razvili majhne sferoidne tumorske modele (8, 9). Namen naše raziskave je zgraditi preprost 3-D model *in vitro*, kjer bomo skušali doseči uspešno gensko elektrotransfekcijo. Preizkusili smo dva različna gela kot 3-D ogrodji, v katerih smo skušali gojiti štiri različne celične linije. *In vitro* analiza različnih parametrov genske elektrotransfekcije v 3-D modelu *in vitro* in teoretična obravnava le-teh lahko pripomorejo k razlagi zaenkrat še neznanih mehanizmov. Prav tako s študijo genske elektrotransfekcije v imitiranem okolju *in vivo* lahko pripomoremo k izboljšanju uspešnosti metode v kliničnih aplikacijah genske terapije, ki so še vedno premalo učinkovite.

2 MATERIALI IN METODE

2.1 Celične kulture

V študiji smo uporabili naslednje transformirane celične linije:

- pljučni fibroblasti kitajskega hrčka − V79,
- celice mišjega melanoma − B16F1,
- humani kožni fibroblasti – 1306,
- celice ovarija kitajskega hrčka – CHO-K1.

Celice V79 in B16F1 smo gojili v gojišču DMEM (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija), celice CHO-K1 pa v gojišču F12 (HAM) (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija). Gojišči smo obogatili s telečjim serumom (FBS; Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija) v končni koncentraciji 10 %, ki smo mu dodali glutamin (10 mM; Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija). Humane kožne fibroblaste smo gojili v mediju EMEM (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija), ki smo ga obogatili s FBS v končni koncentraciji 15 % in ki smo mu dodali 2 mM glutamin. Vsa gojišča so vsebovala antibiotika kristacilin (1 ml/l; Pliva d.d., Zagreb, Hrvaška) in gentamicin (400 µl/l; Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija). Celice smo gojili v inkubatorju Kambič (Semič, Slovenija) pri 37ºC in 5 % atmosferi CO_2. Za poskuse smo uporabljali celice v eksponentni fazi rasti.

2.2 Priprava 3-D *in vitro* ogrodja

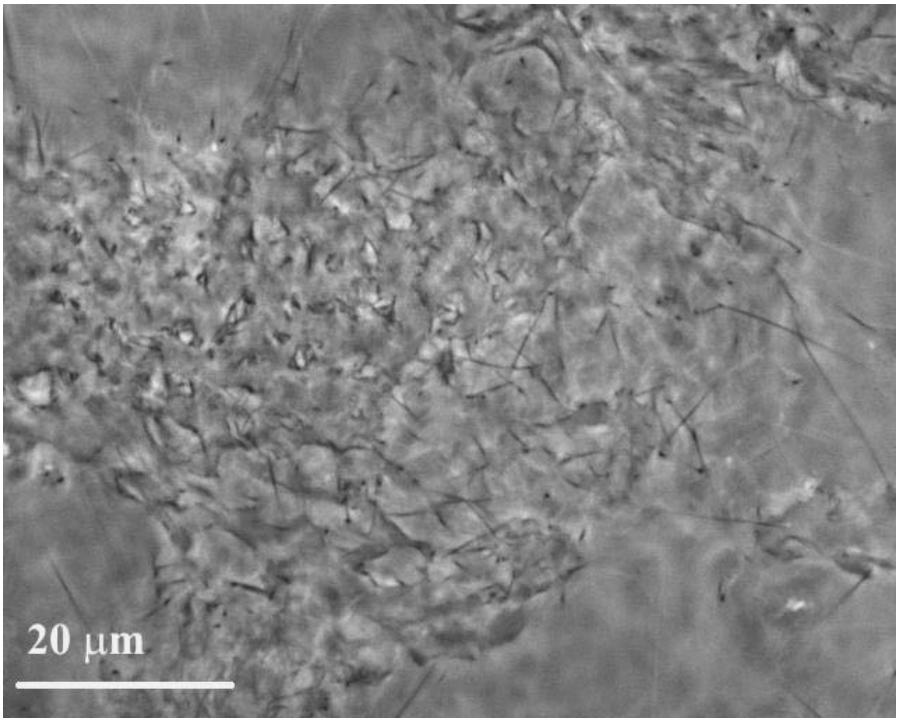
2.2.1 Gel zunajceličnega ogrodja

Komercialno dostopni gel zunajceličnega ogrodja (gel ECM), pridobljenega iz sarkoma miši Engelbreth-Holm-Swarm (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija), smo ponoči odtajali na 4ºC. Odtajani gel ECM smo po priporočilih proizvajalca dvakratno redčili s primernim hladnim gojiščem. Sledila je tripsinizacija celic z uporabo 0.25 % raztopine tripsin/EDTA (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija). Po tripsinizaciji smo celicam dodali primerno gojišče in jih centrifugirali 5 min pri 1.000 obratih/minuto in 4ºC. Celice smo nato resuspendirali v gelu ECM v koncentraciji 2 x 10^5 celic/ml. V vsako vdolbinico mikrotitrske plošče s 24 vdolbinicami smo nanesli 240 µl mešanice celic in gela ECM. Po 1 h inkubacije pri 37ºC in 5 % atmosferi CO_2 se je gel ECM strdil. Previdno smo dodali 1 ml primernega toplega gojišča in posodico s celicami v gelu ECM shranili za 24 h pri 37ºC in 5 % atmosferi CO_2.

2.2.2 Kolagenski gel

Komercialno dostopni kolagen tipa I v kosmičasti obliki, pridobljen iz podganjega repa (Sigma-Aldrich Chemie GmbH, Deisenhofen, Nemčija), smo raztopili v razredčeni ocetni kislini (28.5 ml ocetne kisline/liter vode) do koncentracije 4 mg/ml. Po 24-urni inkubaciji raztopine kolagena pri 4ºC smo pripravili kolagensko mešanico, primerno za rast celic. Raztopini kolagena smo dodali primerno celično gojišče in fosfatni pufer (PBS) v razmerju raztopina kolagena : celično gojišče : PBS = 2.3 : 0.5 : 0.5. pH kolagenske mešanice smo z dodajanjem 0.1 M NaOH uravnali do vrednosti pH = 7.2−7.4. Sledila je tripsinizacija celic z uporabo 0.25 % raztopine tripsin/EDTA. Po tripsinizaciji smo celicam dodali primerno gojišče in jih centrifugirali 5 min pri 1.000 obratih/minuto in 4ºC. Celice smo resuspendirali v kolagenski mešanici v koncentraciji

5.6 x 10^5 celic/ml. V vsako vdolbinico mikrotitrske plošče s 24 vdolbinicami smo nanesli 180 µl mešanice celic in kolagena. Po 2 h inkubacije pri 37ºC in 5 % atmosferi CO_2 so kolagenska vlakna polimerizirala in tvorila gel. Slika 1 prikazuje polimerizirana kolagenska vlakna. Previdno smo dodali 1 ml primernega toplega gojišča in posodico s celicami v kolagenskem gelu shranili za 24 h pri 37ºC in 5 % atmosferi CO_2.



Slika 1: Polimerizirana kolagenska vlakna. Opazovali smo jih z invertnim fluorescentnim mikroskopom − fazni kontrast, pri 1000-kratni povečavi z imerzijskim oljem. Merilo = 20 µm.

2.3 Preživetje celic v 3-D ogrodju *in vitro*

Preživetje celic v 3-D ogrodju *in vitro* smo določili z opazovanjem celične morfologije pod svetlobnim mikroskopom (Olympus Europa Holding GmbH). Nežive celice so izražale značilne morfološke karakteristike, značilne za proces apoptoze (kot so zažetki membrane, fragmentirano jedro) (10).

2.4 Genska elektrotransfekcija

Za ugotavljanje učinkovitosti vnosa gena v ciljne celice, gojene v 3-D celičnem ogrodju, smo uporabili plazmidno DNA, in sicer komercialno dostopen reporterski vektor pGFP-N1 (Clontech Laboratories Inc., Mountain View, Kanada, ZDA), ki kodira zeleno fluorescirajoči protein (GFP; eksitacijski vrh 488 nm, emisijski vrh 507 nm). Za pridobivanje večjih količin plazmidne DNA smo pGFP-N1 transformirali v bakterijski sev *Escherichia coli* DH5α. Bakterije smo čez noč namnožili v 1−2 litrih tekočega medija LB, kateremu smo dodali ustrezen antibiotik (ampicilin, kanamicin). Za izolacijo plazmidne DNA iz bakterij smo uporabili komercialni komplet HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Nemčija) ter koncentracijo določili spektrofotometrično, z merjenjem absorpcije svetlobe pri valovni dolžini 260 nm. Uspešnost izolacije in pravilno velikost plazmidne DNA smo preverili z agarozno gelsko elektroforezo.

Celicam, gojenim v 3-D celičnem ogrodju, smo previdno odstranili gojišče in jim dodali 200 µl standardnega elektroporacijskega fosfatnega medija (pH 7.4, 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$ in 250 mM saharoza), ki je vseboval plazmidno DNA v različni koncentraciji. Po 30 min inkubacije na sobni temperaturi (22ºC) je sledilo dovajanje serije osmih pravokotnih električnih pulzov z različno jakostjo električnega polja, različnim trajanjem pulza in ponovitveno frekvenco 1 Hz. Pri kontrolnih vzorcih nismo dovajali električnih pulzov. Uporabili smo ploščate elektrode iz nerjavečega jekla z razdaljo 4 mm med elektrodama.

Po končanem dovajanju električnih pulzov smo celicam dodali 70 µl telečjega seruma (FBS; Sigma, St. Louis, ZDA), da bi omogočili boljše preživetje. Celice smo inkubirali 15 min pri 37ºC in 5 % atmosferi CO_2, jim nato dodali 1 ml ustreznega gojišča in jih za 24 h shranili pri 37ºC in 5 % atmosferi CO_2.

2.5 Elektroporator

Za dovajanje električnih pulzov smo uporabili elektroporator Jouan GHT 1287 (Jouan, St. Herblain, Francija). Napetost in tok smo opazovali z osciloskopom Wave surfer^{TM} 422 (LeCroy, Chestnut Ridge, New York, ZDA).

2.6 Detekcija genske elektrotransfekcije

Gensko elektrotransfekcijo (spremljanje izražanja gena GFP v celicah) smo določali z invertnim fluorescentnim mikroskopom Axiovert 200 (Zeiss, Nemčija). Za vzbujanje smo uporabili Xenonovo obločnico z monokromatorjem (PolyChrome IV, Visitron, Nemčija) in nastavili vzbujevalno svetlobo na valovno dolžino 488 nm. Uporabili smo objektiv z desetkratno ali dvajsetkratno povečavo in filter, ki je prepuščal svetlobo valovne dolžine 507 nm (za GFP). Slike smo zajeli z digitalno kamero VISICAM 1280 (Visitron, Nemčija) in jih analizirali s programom MetaMorph 5.0 (Visitron, Nemčija).

3 REZULTATI IN RAZPRAVA

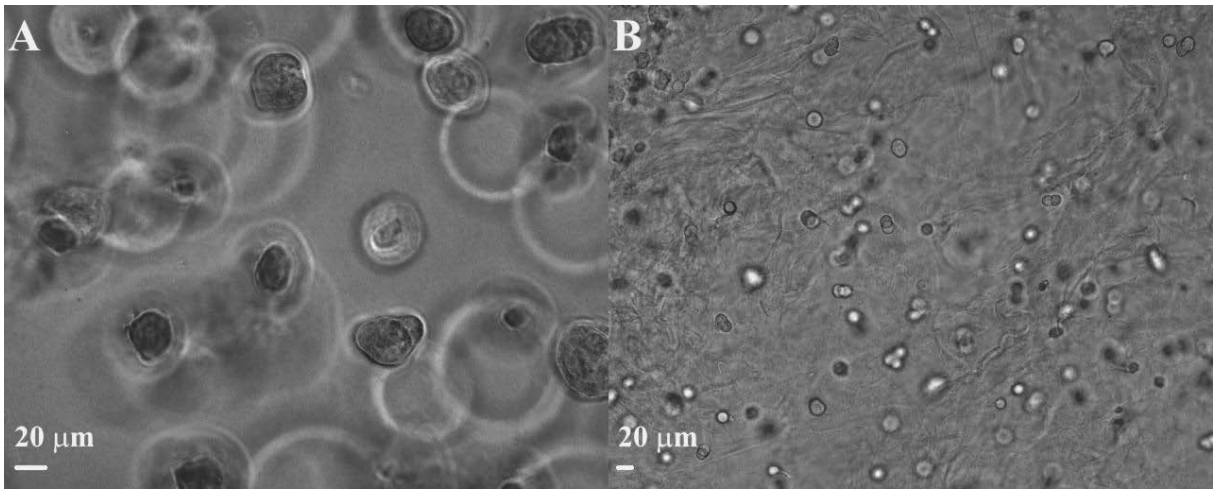
Naš namen je bil zgraditi preprost 3-D model *in vitro*, kjer bodo celice preživele in bomo uspeli doseči učinkovito gensko elektrotransfekcijo. Zato smo preizkusili dve različni 3-D ogrodji - gel ECM in kolagenski gel, v katerih smo poskusili gojiti štiri različne celične linije. Po 24 h inkubacije pri 37ºC in 5 % atmosferi CO_2 smo s pomočjo opazovanja njihove morfologije pod svetlobnim mikroskopom ugotavljali njihovo preživetje.

Pri celicah, ki so preživele v izbranih 3-D celičnih ogrodjih, smo z metodo genske elektrotransfekcije skušali vnesti plazmidno DNA.

3.1 Preživetje celic v 3-D ogrodju *in vitro*

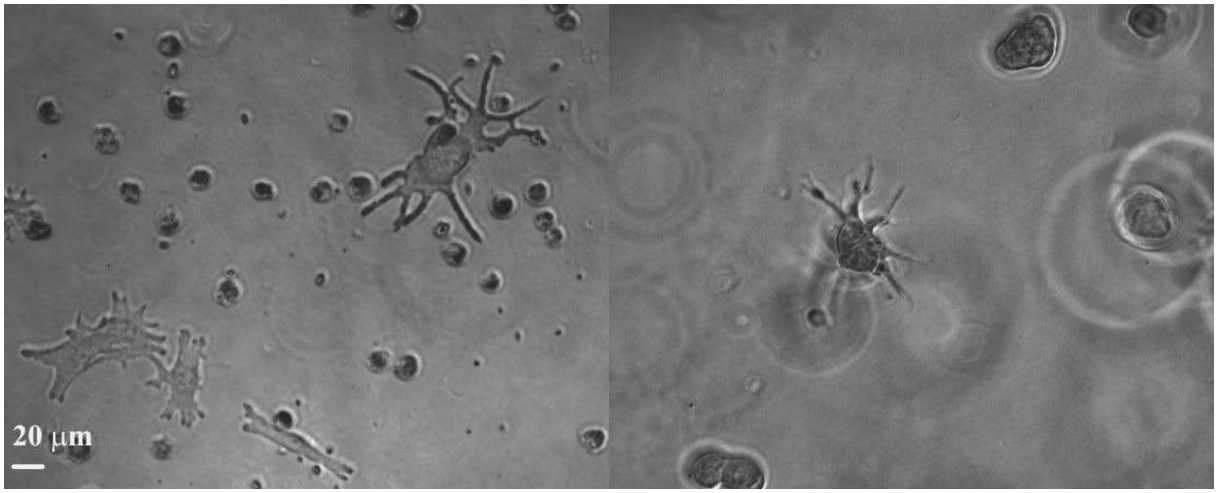
Po 24 h inkubacije pri 37ºC in 5 % atmosferi CO_2 smo opazovali preživetje celic v 3-D ogrodjih. Celice, ki niso preživele so imele na svoji površini membranske zažetke, značilne za proces apoptoze.

a) Celice V79 − v 3-D ogrodju iz gela ECM ali iz kolagenskega gela nismo opazili živih celic.
b) Celice B16F1 − opazili smo žive celice v obeh 3-D ogrodjih. Celice so bile sferične oblike in se niso vraščale v 3-D ogrodje iz gela ECM (Slika 2A) ali iz kolagenskega gela (Slika 2B).



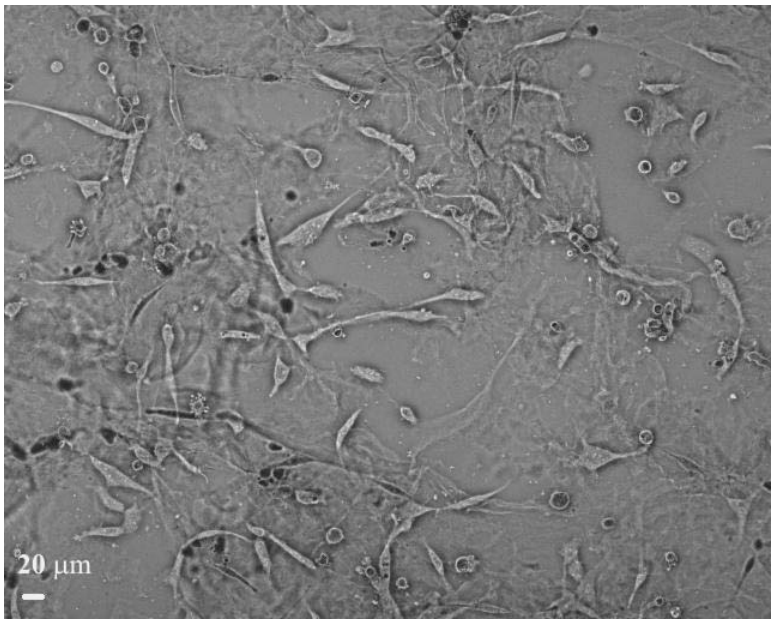
Slika 2: Celice B16F1 v 3-D modelu *in vitro* iz (A) gela ECM – 200-kratna povečava − in (B) kolagenskega gela – 100-kratna povečava. Celice smo opazovali pod faznim kontrastom. Merilo = 20 μm.

Opazili smo tudi redke, posamezne celice zvezdastih oblik, ki so se vpenjale v 3-D strukturo iz gela ECM (Slika 3).



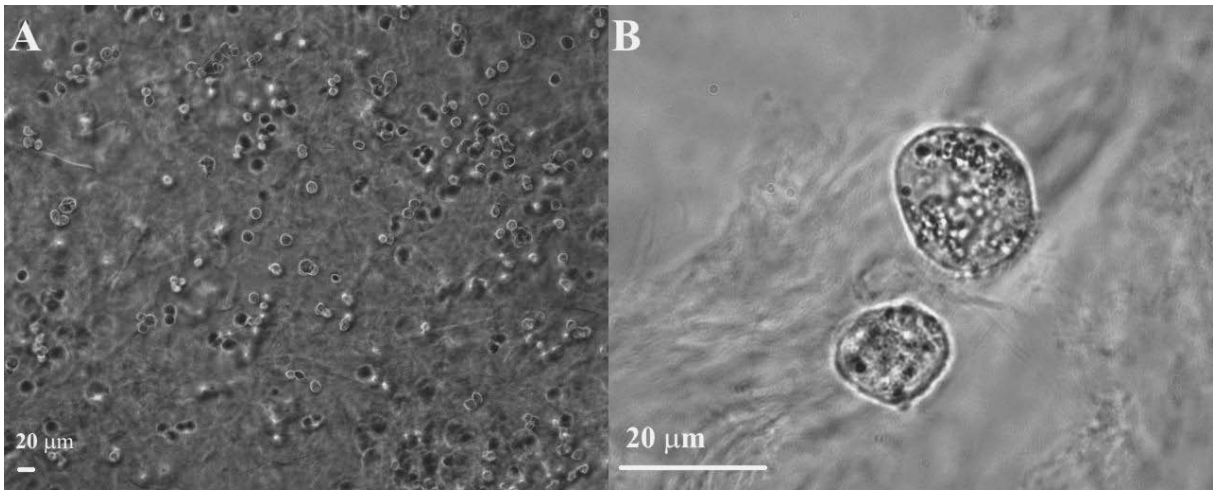
Slika 3: Celice B16F1 v 3-D modelu *in vitro* iz gela ECM. Celice smo opazovali pod faznim kontrastom, pri 200-kratni povečavi. Merilo = 20 μm.

c) Celice 1306 − v 3-D ogrodju iz gela ECM nismo opazili živih celic, medtem ko smo v 3-D ogrodju iz kolagenskega dela opazili večinoma žive celice. Celice so bile tako sferične oblike, te se niso vraščale v 3-D strukturo, kot podolgovate oblike, ki pa so se vraščale v 3-D strukturo (Slika 4).



Slika 4: celice 1306 v 3-D modelu *in vitro* iz kolagenskega gela. Celice smo opazovali pod faznim kontrastom, pri 100-kratni povečavi. Merilo = 20 μm.

d) Celice CHO-K1 − v 3-D ogrodju iz gela ECM nismo opazili živih celic, medtem ko smo v 3-D ogrodju iz kolagenskega gela opazili žive celice. Celice so bile predvsem sferične oblike in se niso vraščale v 3-D ogrodje (Slika 5).



Slika 5: Celice CHO-K1 v 3-D modelu *in vitro* iz kolagenskega gela. Celice smo opazovali pod faznim kontrastom, pri (A) 100-kratni in (B) 1000-kratni povečavi z imerzijskim oljem. Merilo = 20 μm.

Izražanje celičnih membranskih integrinov ima pomembno vlogo pri komunikaciji celice z okoljem (11). Predvsem naj bi mehansko celično zaznavanje njenega okolja imelo pomembno vlogo pri regulaciji preživetja celice (12).

Delo s celičnimi linijami, ki niso preživele v izbranem 3-D ogrodju ali pri katerih smo imeli večkratne težave s preživetjem, smo opustili (celična linija V79). Ker so 3-D ogrodja dovolj porozna za izmenjavo plinov in hranil, sklepamo, da celice, ki niso preživele v 3-D ogrodjih, niso izražale primernih integrinov oziroma verjetno niso tvorile napetostnih sil, potrebnih za rast v takšnem okolju (12 , 13).

3.2 Genska elektrotransfekcija

Glede na uspešnost preživetja v 3-D modelih smo se osredotočili na vnos plazmidne DNA z dovajanjem električnih pulzov v celice B16F1, 1306 in CHO-K1. Uspešnost

genske elektrotransfekcije smo določali 24 h po elektroporaciji s fluorescentnim invertnim mikroskopom:

a) Celice B16F1 − celicam smo dodali elektroporacijski fosfatni medij, ki je vseboval plazmidno DNA v koncentraciji 90 μg/ml, in dovedli serijo osmih pulzov, trajanja 1 ms, jakosti električnega polja 0.8 kV/cm in s ponavljalno frekvenco 1 Hz. 24 h po dovajanju električnih pulzov nismo opazili uspešnega vnosa plazmidne DNA v celice B16F1, gojene v 3-D modelu *in vitro* iz gela ECM ali iz kolagenskega gela.

b) Celice 1306 − celicam smo dodali elektroporacijski fosfatni medij, ki je vseboval plazmidno DNA v koncentraciji 90 μg/ml, in dovedli serijo osmih pulzov, trajanja 1 ms, jakosti električnega polja 0.8 kV/cm in s ponavljalno frekvenco 1 Hz. 24 h po dovajanju električnih pulzov smo opazili zelo malo uspešno transfeciranih celic.

c) Celice CHO-K1 − uspešnost genske elektrotransfekcije (približno 6 %) je opisana v Poglavju 3 (14).

Namen naše raziskave je bil razviti preprost 3-D model *in vitro,* kjer bomo pokazali možnost vnosa DNA v celice z dovajanjem električnih pulzov. Delo s celicami, ki v 3-D ogrodjih niso preživele (V79) smo opustili.

Sledili so poskusi transfekcije celic (B16-F1, 1306 in CHO-K1) gojenih v 3-D ogrodjih z dovajanjem električnih pulzov. Pri tem smo uspešno vnesli plazmidno DNA le v celice CHO-K1, gojene v kolagenskem gelu. Naši rezultati genske elektrotransfekcije so bili primerljivi z rezultati genske elektrotransfekcije *in vivo,* zato smo delo z izgrajenim celičnim modelom nadaljevali (Poglavje 3 in 4).

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Poglavje 4

Analiza mobilnosti DNA in genske elektrotransfekcije v 3-D *in vitro* kolagenskem modelu

Analysis of DNA mobility and gene electrotransfer in 3-D *in vitro* collagen gel model

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V pripravi za objavo

POVZETEK

Genska elektrotransfekcija je obetavna nevirusna metoda, ki se uporablja za vnos plazmidne DNA v celico z dovajanjem električnih pulzov. V številnih študijah v okoljih *in vitro* in *in vivo* so analizirali različne dolžine električnih pulzov, vendar mehanizem genske elektrotransfekcije še vedno ostaja nepojasnjen. Ena izmed poglavitnih ovir za uspešno gensko elektrotransfekcijo v okolju *in vivo* predstavlja slaba mobilnost DNA v tkivu. V naši študiji smo analizirali vpliv slabe mobilnosti DNA na uspešnost genske elektrotransfekcije. V ta namen smo pritrjenim celicam, celicam, gojenim na kolagenskem gelu, in celicam, vpetim v gel iz kolagena (3-D model), dovajali različno trajajoče električne pulze in preučili učinkovitost vnosa DNA in preživetje celic. Najvišji vnos DNA smo dosegli na pritrjenih celicah, medtem ko je bila genska elektrotransfekcija v 3-D modelu najnižja in primerljiva z rezultati, pridobljenimi *in vivo*. Nadalje smo analizirali slabo mobilnost DNA v 3-D modelu, tako da smo DNA nanesli na površino 3-D modela ali jo vnesli z injiciranjem v 3-D strukturo. Pokazali smo, da slednji način omogoča višjo gensko elektrotransfekcijo, kar so tudi pokazali s študijami *in vivo*. Prav tako smo testirali vpliv različnih kombinacij visokonapetostnih, nizkonapetostnih in iz različnih smeri dovedenih pulzov na učinkovitost genske elektrotransfekcije v 3-D modelu. Predlagamo, da naš preprost 3-D model predstavlja okolje, ki je, v nasprotju s klasično 2-D celično kulturo, podobno okolju *in vivo* in nam omogoča analizo mehanizmov genske elektrotransfekcije.

Ključne besede: 3-D model *in vitro*, kolagenski gel, genska elektrotransfekcija, GFP, visoko napetostni pulz, nizkonapetostni pulz, obračanje smeri pulza.

ABSTRACT

Gene electrotransfer is a promising nonviral method that enables transfer of plasmid DNA into cells with electric pulses. Several *in vitro* and *in vivo* studies analyzed different pulse durations however the question of the mechanisms involved in gene electrotransfer remains open. One of main obstacles toward efficient gene electrotransfer *in vivo* is relatively poor mobility of DNA in tissues. In order to analyze the effect of impaired mobility on gene electrotransfer efficiency, we applied electric pulses with different durations on plated cells, cells grown on collagen layer and cells embedded in collagen gel (3-D model) and compared gene electrotransfer efficiency and viability of cells. We obtained the highest transfection of plated cells, while transfection efficiency of embedded cells in 3-D model was lowest and similar as in *in vivo*. To further analyze poor DNA mobility in 3-D model, we applied DNA in top of or injected it into 3-D model and showed that former way increases gene electrotransfer efficiency as was shown in *in vivo* studies. Also different combinations of high-voltage and low-voltage pulses and pulses with different polarities were used in order to analyze their effect on gene transfer efficiency. We suggest that our 3-D collagen model resembles the *in vivo* situation more closely than conventional 2-D cell cultures and thus provides an intermediate between *in vitro* and *in vivo* conditions to study mechanisms of gene electrotransfer.

Keywords: 3-D *in vitro* model, collagen gel, gene electrotransfer, GFP, high-voltage pulse, low-voltage pulse, different polarity pulses.

1 INTRODUCTION

Gene therapy represents a forefront of medicine in order to heal several diseases, which could not be cured by conventional treatment. It enables the insertion of healthy genes or alteration or removal of defective genes responsible for disease development (1). The most common method used for gene therapy is using a viral vector that has been genetically altered to carry normal human DNA (2). Although viral vectors have been very efficient, the safety of their use has been questioned (3). Thus, there is a great interest in developing non-viral methods for gene delivery. For the past 20 years a huge variety of non-viral gene therapy methods, including chemical and physical ones, have been developed to introduce DNA into the cell *in vivo*, but many of them are either toxic or have poor gene expression (4, 5, 6).

Almost four decades ago a physical method for delivery of molecules by use of electric pulses (electroporation) was described (7). It is based on the significant increase in the permeability of the cell plasma membrane caused by an externally applied electrical field. The method is already successfully applied in different biomedical applications, including: electrofusion (8, 9); electrochemotherapy (10, 11, 12, 13); irreversible tissue ablation (14); DNA vaccination (15) and gene electrotransfer (16, 17). Today gene electrotransfer is widely used to introduce DNA into different cells (18, 19) and tissues (17, 20, 21) due to its efficiency, safety and easy application. It is also relevant in a variety of clinical settings including cancer therapy, modulation of pathogenic immune responses, delivery of therapeutic proteins and drugs (22). Although the mechanisms of gene electrotransfer are not yet fully understood, it was shown that several steps are needed for successful transfection: (i) migration of DNA towards the cell; (ii) DNA insertion into the cell membrane; (iii) translocation across the membrane; (iv) migration of DNA towards the nucleus; (v) transfer of DNA across the nuclear envelope and finally (vi) gene expression (23).

Many parameters have been described, which can influence the efficiency of gene electrotransfer *in vitro* (18, 24, 25 - 37) and *in vivo* (20, 38 - 46). A number of *in vivo* published reports have concentrated especially on optimization of electric pulse protocol. At first, only low voltage electric pulses with long duration were used (47). More recent studies have shown that transfection can be achieved by using the

combination of high-voltage (HV) short duration pulse, followed by a different number of low-voltage (LV) long duration electric pulses (23, 39, 48 - 50). It was suggested that HV pulses are crucial for permeabilization of cell membrane, while LV pulses electrophoretically drag DNA to the cell. It was also demonstrated, that LV pulse is crucial in a condition where there is low concentration of plasmid (i. e. *in vivo*) (24). However, *in vivo* studies require large quantity of sacrificed animals, thus 3-D *in vitro* tissue models could be used to study gene electrotransfer (51, 52). We previously developed a simple 3-D model composed of collagen gel, where successful gene electrotransfer was shown (52).

In this study we further analyzed the effect of impaired DNA mobility inside 3-D collagen model, since one of the main problems of *in vivo* gene electrotransfer is slow diffusion of DNA in tissue (53). Close contact of DNA with the cell membrane at the moment of pulse application is crucial for efficient delivery (27). Extracellular matrix, which surrounds the cells in tissues, hinders DNA mobility. Consequently only small fraction of cells is in contact with DNA (20, 54) and overall gene electrotransfer efficiency is decreased.

The aim of our study was to analyze, how slow mobility of DNA and different pulsing protocols in collagen 3-D model influences gene electrotransfer efficiency.

To analyze impaired DNA mobility, we first grew cells as a monolayer culture, on top of collagen layer or we embedded cells into 3-D model. For these protocols DNA was always applied on top of cells. To further show how slow DNA mobility in 3-D collagen model influences gene electrotransfer efficiency, we applied DNA on top of or injected it into the 3-D model.

Also the effect of HV and LV pulses on the efficiency of gene transfer in 3-D model was investigated, since LV pulses were shown to improve gene electrotransfer efficiency *in vitro* (24, 55) and *in vivo* (23, 39, 48 - 50, 56).

Since changing the polarity of electric field during the electric pulse delivery is also important for gene electrotransfer as it allows interaction of DNA molecules on many sides of the cell membrane perpendicular to direction of electric field (25), we further investigated the effect of single polarity and orthogonal both polarities pulses on gene transfer in 3-D model.

2 MATERIALS AND METHODS

2.1 Cell culture

For the experiment Chinese hamster ovary cells (CHO-K1) were used (European Collection of Cell Cultures, Salisbury, UK). Cells were: (i) grown as a monolayer culture in 24-multiwell plate, (ii) grown on top of collagen gel layer and (iii) embedded in collagen gel (3-D model). For cells, culture medium F-12 HAM (Dulbecco's modification of EMEM) supplemented with 10% fetal bovine serum and 0.15 mg/ml L-glutamine (Sigma-Aldrich, St. Louis, MO) was used.

2.1.1. Preparation of cells grown as a monolayer culture

CHO-K1 cells were plated as a monolayer culture in Ham's tissue culture medium in 24-multiwell plate in cell density of $\rho = 5 \times 10^4$ cells/ml. The plate was stored for 24 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator (Kambič, Slovenia).

2.1.2. Preparation of cells grown on top of collagen gel layer

Type I collagen from rat tail was obtained from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) as a powder and mixed with diluted acetic acid (28.5 ml glacial acetic acid/litter) to achieve collagen solution concentration 4.0 mg/ml and stored at 4°C. After 24 h 1x PBS, pH=7.4 was added to collagen solution, in the ratio of 1:8. pH of mixture was adjusted to 7.2-7.6 with 0.1 M NaOH. To prevent gelation, temperature of mixture was maintained at 2–8°C. 200 µl of collagen was pipeted into each space of 24-multiwell plate and stored for 1 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator. Collagen polymerized and formed a gel layer.

After 1 h incubation of collagen layer at 37°C, CHO-K1 cells were added on top of collagen layer as a monolayer culture in Ham's tissue culture medium in cell density of $\rho = 5 \times 10^4$ cells/ml. The plate was placed back into the incubator (37°C, 5% CO_2) for 24 h.

2.1.3. Preparation of collagen gel with embedded cells (3-D model)

Collagen solution was prepared as described above. After 24 h incubation of collagen solution at 4°C, collagen mixture was prepared as already described before (52). Briefly, 2.3 parts of chilled collagen solution was mixed with 0.5 part of Ham tissue culture medium for mammalian cells and 0.5 part of 1x PBS, pH=7.4. CHO-K1 were prepared as a cell suspension and cell pellet was resuspended with liquid collagen solution to a cell density of $\rho = 5.6 \times 10^5$ cells/ml. 180 µl of collagen with cells was pipeted into each space of multiwell dish and stored for 1 h at 37°C in a humidified 5% CO_2 atmosphere in the incubator. After raising the temperature to 37°C collagen polymerized and formed a gel with embedded cells inside (3-D model). Ham's tissue culture medium was gently added and cells were stored for 24 h at 37°C in a humidified 5% CO_2 atmosphere.

2.2 Plasmid DNA

Plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) encoding green fluorescent protein (GFP) was amplified in DH5α strain of Escherichia coli and isolated with HiSpeed Plasmid Maxi Kit (Qiagen, Hilden, Germany). Plasmid DNA concentration was spectrophotometrically determined at 260 nm and confirmed by gel electrophoresis.

2.3 Gene electrotransfer

Our study was divided into three sets of experiments. In the first part, gene electrotransfer was performed on plated cells, on cells grown on top of collagen layer and on cells embedded in 3-D model. In the second part gene electrotransfer was performed on cells embedded in 3-D model, where DNA was applied on top or injected into the 3-D model and in the third part we analyzed gene electrotransfer efficiency in 3-D model by using different pulsing protocols (combinations of high-voltage and low-voltage pulses, single polarity pulses and orthogonal both polarities pulses).

Electroporation was performed on 24 h old cell culture with standard electroporation media (pH 7.4, 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM $MgCl_2$ and 250 mM sucrose).

On the day of the experiment culture medium was removed and cells were incubated with 200 µl of electroporation media with plasmid DNA that codes for GFP for 30 min at a room temperature (22°C). Plasmid DNA concentration in electroporation media was 90 µg/ml.

In first two parts of the experiment for pulsing Jouan GHT 1287 electroporator (Jouan, St. Herblain, France) and an oscilloscope Wave surfer™ 422 (Le croy, Chestnut Ridge, New York, USA) to monitor pulse shape were used. The distance between a pair of two plate stainless steel parallel electrodes was d = 4 mm.

In third part of the experiment for pulsing pulse generator Cliniporator™ (IGEA s.r.l., Carpi, Modena, Italy) was used which enabled different combinations of high- (HV) and low-voltage (LV) pulse. The distance between a pair of two plate stainless steel parallel electrodes was d = 4 mm. For analyzing gene electrotransfer efficiency by changing pulse polarity a high-voltage prototype generator (EP-GMS 7.1) was used (57), which allowed application of relatively homogeneous electric field in different directions. An oscilloscope Wave surfer™ 422 (Le croy, Chestnut Ridge, New York, USA) monitored pulse shape. Especially designed electrodes allowing delivery of electric field in different directions and at the same time providing relatively homogeneous electric field distribution were used (57). No electric pulses were applied to cells in a control sample.

In the first part of the experiment, electroporation media with plasmid DNA was applied on top of plated cells, cells grown on top of collagen layer and cells embedded in 3-D model. A train of eight square wave pulses of different pulse durations: 200 µs, 1 ms and 5 ms were used to deliver DNA into the cells. Electric field strength was 0.8 kV/cm, with repetition frequency 1 Hz for all pulsing protocols.

In the second part, two ways of DNA application was studied in 3-D model: (i) electroporation media with plasmid DNA applied on top of 3-D model or (ii) electroporation media with plasmid DNA injected into the 3-D model. Electric pulses of two different pulse durations were used: 8 x 1 ms and 8 x 2 ms to deliver DNA into the

cells. Electric field strengths used were 0.6 kV/cm, 0.8 kV/cm and 1.0 kV/cm, with repetition frequency 1 Hz for all pulsing protocols.

In the third part, electroporation media with plasmid DNA was applied on top of cells embedded in 3-D model. Different types of pulsing protocols were used to deliver DNA into the cells as shown in Table 1 and Figure 1.

Table 1: Pulsing protocols for gene electrotransfer in 3-D model. The time lag between HV and LV pulse was always 20 ms.

Electric pulse parameters
HV 1 (5 x 1 ms; 0.8 kV/cm; 1 Hz)
HV 2 (8 x 200 μs; 0.8 kV/cm; 1 Hz)
LV 1 (1 x 100 ms; 75 V/cm)
LV 2 (1 x 100 ms; 150 V/cm)
Single polarity pulses-SP (8 x 1 ms; 0.8 kV/cm; 1 Hz)
Orthogonal both polarities pulses-OBP (8 x 1 ms; 0.8 kV/cm; 1 Hz)

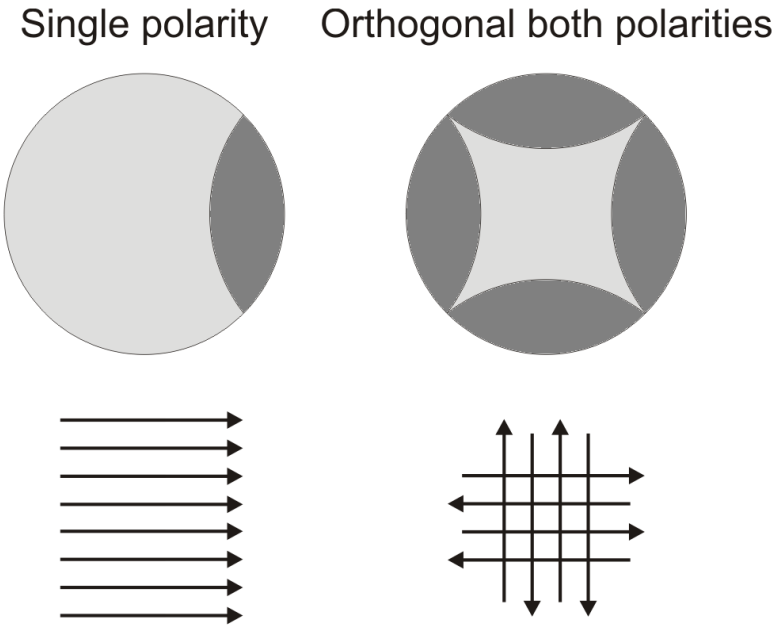

Figure 1: In single polarity (SP) electric pulses are applied between two opposite electrodes. While in orthogonal both polarities (OBP) electric pulses are applied between both opposite pairs of electrodes.

After exposing cells to electric pulses, 70 µl of fetal calf serum was added (35% of sample volume) to preserve cell viability. Cells were then incubated for 15 min at 37°C to allow cell membrane resealing and then grown for 24 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in the incubator.

Gene electrotransfer efficiency was determined by fluorescent microscopy (Zeiss 200, Axiovert, ZR Germany) with excitation wavelength at 488 nm generated with a monochromator system (PolyChrome IV, Visitron, Germany) and emission was detected at 507 nm. The images were recorded using imaging system (MetaMorph imaging system, Visitron, Germany). At least ten fluorescence images were acquired in the area between the electrodes at 10× objective magnification per each parameter. The cells were counted manually and gene electrotransfer efficiency was determined by the ratio between the number of green fluorescent cells (successfully transfected) and the total number of cells. Three independent experiments were performed for each parameter and results are presented as a mean values±standard deviation.

2.4 Cell viability

Cell viability was determined by measuring propidium iodide (PI) uptake 24 h after applying pulses as was already described (52). Briefly, culture medium was removed and 200 µl of PBS with 6 µl of 0.15 mM PI was added to cells. After 5 min incubation cell viability was determined by fluorescent microscopy. The cells were counted manually under the fluorescent microscope and cell viability was determined by the ratio between the number of dead cells (cells with incorporated PI) and the total number of cells.

3 RESULTS

In our previous study we already developed a simple 3-D model made of collagen gel with embedded cells, where successful gene electrotransfer was obtained (52). In this study in order to asses the influence of DNA mobility on gene electrotransfer efficiency, cells were: (i) plated as a monolayer culture; (ii) grown on top of collagen layer and

(iii) embedded in collagen gel (3-D model). The effect of different pulse duration on gene electrotransfer efficiency (Fig. 2) and cell viability (Fig. 3) was analyzed.

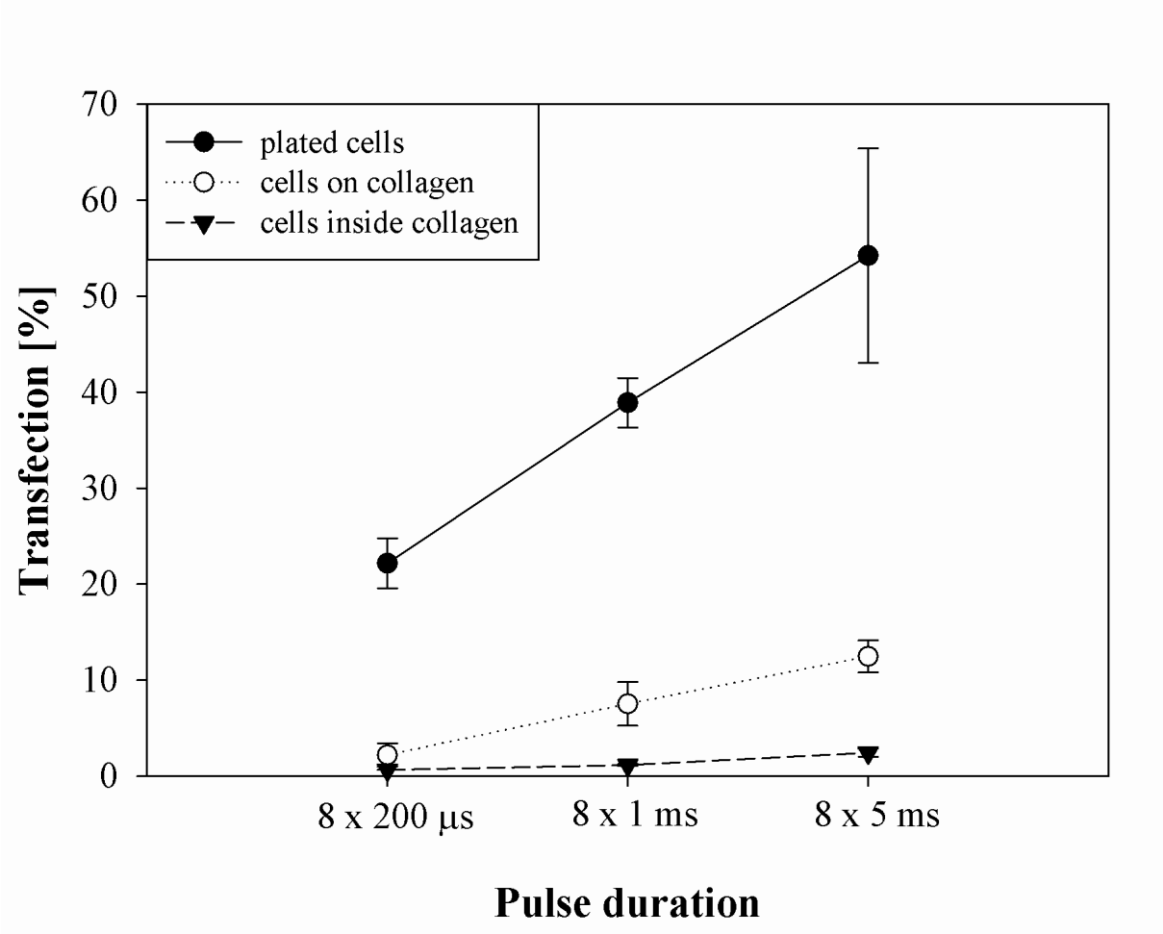

Figure 2: Effect of different pulse duration on gene electrotransfer for: (●) plated cells; (○) cells grown on top of collagen layer; (▼) cells embedded in collagen gel (3-D model). Eight pulses of different durations, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. The percentage of transfected cells is plotted as a function of different pulse durations. Plasmid concentration in electroporation media was 90 µg/ml.

Fig. 2 shows the percentage of transfection (gene electrotransfer efficiency) for different pulse durations of plated cells, of cells grown on top of collagen layer and of 3-D model. We observed that gene electrotransfer efficiency was always significantly higher, when cells were plated as a monolayer culture. Also more cells were successfully transfected when they were grown on top of collagen layer compared to cells in 3-D model. The highest efficiency was obtained when we applied 8 x 5 ms long

pulses. Under this condition 54.2% of viable plated cells, 12.5% of viable cells grown on top of collagen layer and 2.5% of viable cells in 3-D model were transfected.

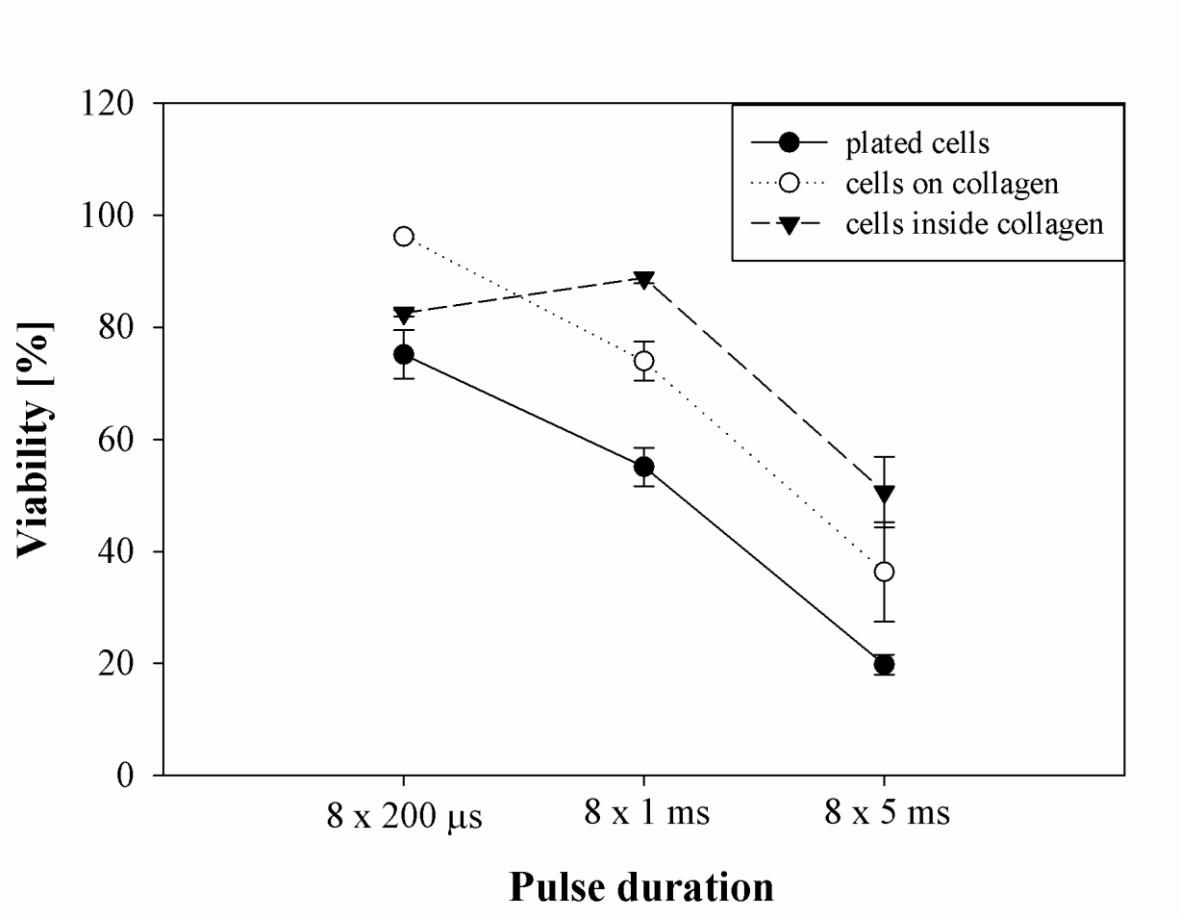

Figure 3: Effect of different pulse duration on viability for: (●) plated cells; (○) cells grown on top of collagen layer; (▼) cells embedded in collagen gel (3-D model). Eight pulses of different durations, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm were applied. The percentage of viable cells is plotted as a function of different pulse durations. Plasmid concentration in electroporation media was 90 µg/ml.

Fig. 3 shows percentage of viable cells grown as plated culture, grown on top of collagen layer and in 3-D model for different pulse durations. The viability of cells embedded in 3-D model was for almost all conditions higher compared to plated cells or cells grown on top of collagen layer. The highest viability was observed when we applied shorter pulses (8 x 200 µs). Under this condition 75% of plated cells, 96% of cells grown on top of collagen layer and 82% of cells embedded in 3-D model survived.

At longer pulses viability of cells was significantly lower. At 8 x 5 ms 19.8% of plated cells, 36% of cells grown on top of collagen layer and 50% of cells embedded in 3-D model survived.

To further analyze how mobility affects gene electrotransfer efficiency, we compared gene electrotransfer efficiency for two cases: (i) electroporation media with DNA was applied on top of 3-D model; (ii) electroporation media with DNA was injected into 3-D model. For all experiments repetition frequency of 1 Hz was used. Fig. 4 shows the percentage of transfection (gene electrotransfer efficiency) for different pulse parameters when DNA was applied on top or injected into 3-D model. In general, for both ways of DNA application the increase in gene electrotransfer efficiency was observed when longer pulses or pulses with higher E were used. For DNA injected into 3-D model we always obtained higher gene electrotransfer efficiency compared to DNA applied on top of 3-D model. The highest efficiency for both ways of DNA application was obtained when we applied 8 x 2 ms long pulses with E = 1.0 kV/cm. Under this condition 6% of viable cells when DNA was injected into 3-D model and 4% of viable cells when DNA was applied on top of 3-D model were transfected.

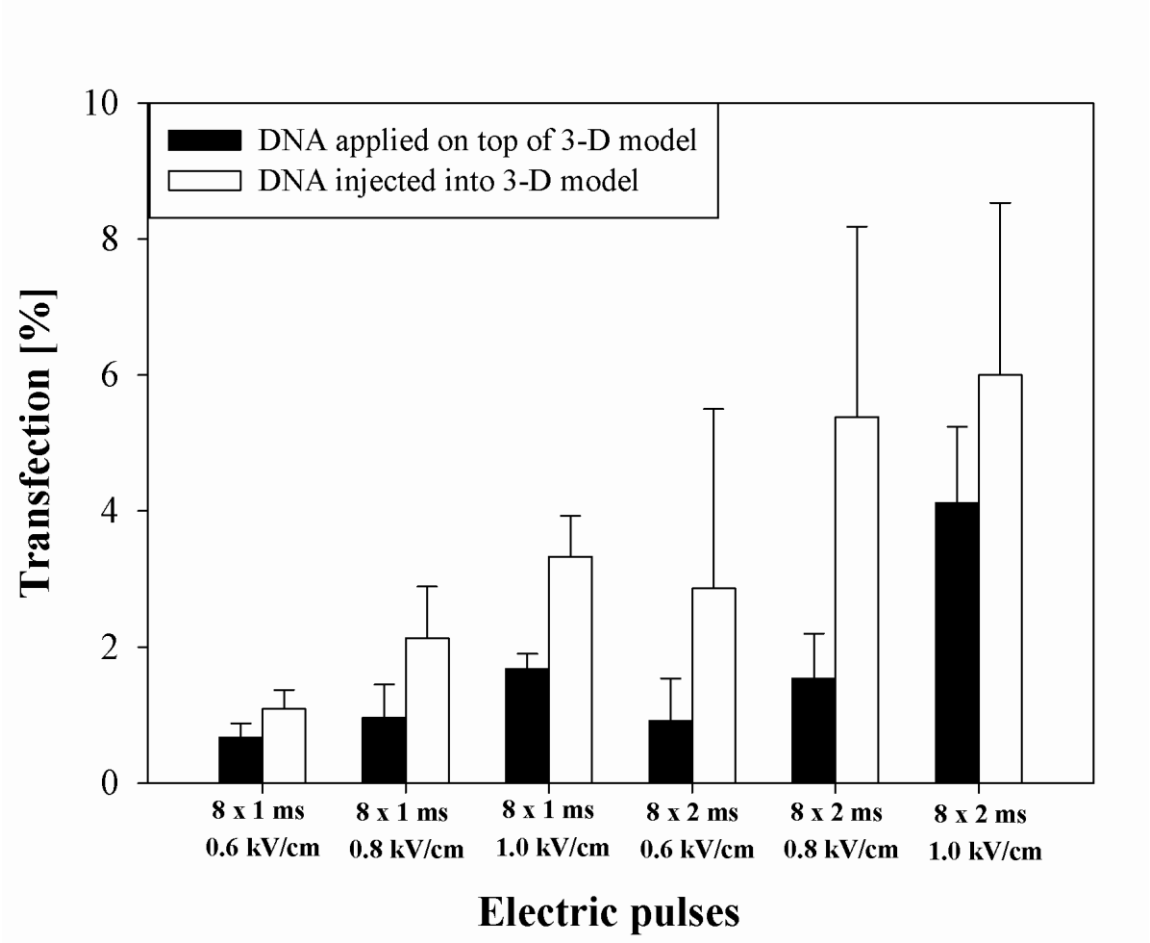

Figure 4: Effect of different pulse durations on gene electrotransfer when: (■) DNA was applied on top of 3-D model; (□) DNA was injected into 3-D model. Eight pulses of 1 ms and 2 ms long, pulse repetition frequency of 1 Hz and different *E* were applied. The percentage of transfected cells is plotted as a function of different electric pulses used. Plasmid concentration in electroporation media was 90 μg/ml. Results are presented as a mean and vertical bars represent standard deviation.

In order to analyze the effect of high-voltage (HV) and low-voltage (LV) pulses on gene electrotransfer efficiency in a 3-D model, we used different combinations of HV and LV pulses (see Table 1). Also pulses with different polarities were used (see Table 1) to evaluate their role on gene transfer in a 3-D model.

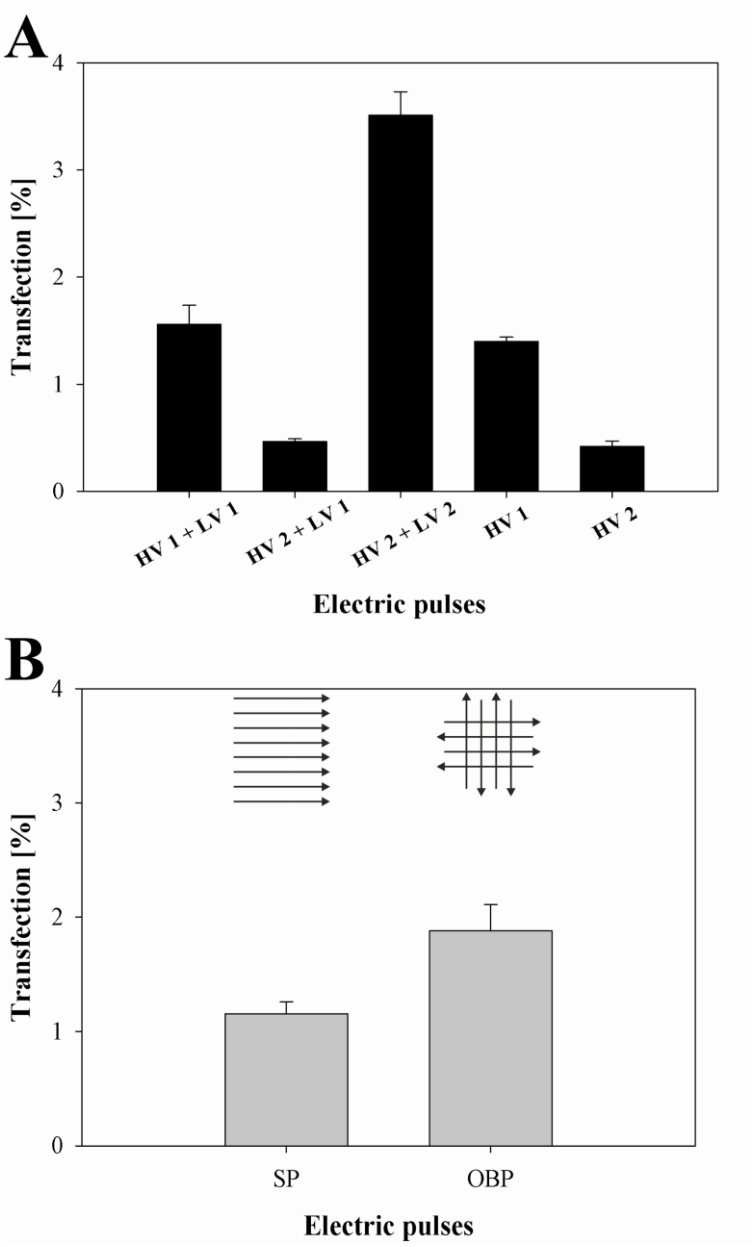

Figure 5: Effect of different pulsing protocols on the efficiency of gene electrotransfer in 3-D model. (A) Different combinations of high-voltage (HV) and low-voltage (LV) pulses were applied. The number and duration of pulses, field strength and repetition frequency were as follows: HV 1 (5 x 1 ms; 0.8 kV/cm; 1 Hz), HV 2 (8 x 200 μs; 0.8 kV/cm; 1 Hz), LV 1 (1 x 100 ms; 75 V/cm) and LV 2 (1 x 100 ms; 150 V/cm). The time lag between HV and LV pulse was always 20 ms. (B) Eight pulses of 1 ms long, pulse repetition frequency of 1 Hz and E = 0.8 kV/cm with single polarity (SP) or orthogonal both polarities (OBP) were applied; The percentage of transfected cells is plotted as a function of different electric pulses used. Plasmid concentration in electroporation media was 90 μg/ml. Results are presented as a mean and vertical bars represent standard deviation.

In Fig. 5A gene electrotransfer efficiency is presented for different combinations of HV and LV pulse protocols. When five 1 ms pulses (HV 1), with *E* = 0.8 kV/cm and 1 Hz repetition frequency were applied, LV pulse (LV 1) did not significantly contribute to gene transfection. Therefore we applied again HV 1 pulse and increased electric field strength of LV pulse from 0.75 V/cm to 150 V/cm (LV 2). At those conditions cell viability was severely reduced and as a consequence gene electrotransfer efficiency was close to zero (data not shown). Afterwards we reduced the duration of HV pulse in order to preserve cell viability. Eight 800 μs long pulses (HV 2) with *E* = 0.8 kV/cm and 1 Hz repetition frequency were applied in combination with LV 1 or LV 2 pulse. It can be seen that when HV 2 pulse with increasing electric field strength of LV pulse was applied, gene electrotransfer efficiency also increased. Maximum gene electrotransfer efficiency was obtained when using HV 2 + LV 2 pulsing protocol (8 x 200 μs; 0.8 kV/cm; 1 Hz + 1 x 100 ms; 150 V/cm), where approximately 3.5% of cells in 3-D model were successfully transfected. The cell viability was around 50% for those parameters (results not shown). With only LV pulse, no transfection was obtained (data not shown).

In Fig. 5B gene electrotransfer is presented for pulses with single or orthogonal both polarities. The higher gene transfer in 3-D model was obtained, when pulses with different polarity were used (OBP) as already shown *in vitro* (57). For those conditions 1.88% of cells in 3-D model were successfully transfected. The cell viability was around 75% for both pulse protocols-SP and OBP (results not shown).

4 DISCUSSION

Gene electrotransfer is an established method to deliver genes both *in vitro* and *in vivo*. The main problem in gene electrotransfer of mammalian cells *in vivo* is currently its relatively low efficiency (53). *In vitro* the DNA can easily reach cells and is therefore directly in contact with the cell membrane, which is one of the crucial steps in gene electrotransfer, while *in vivo*, extracellular matrix hinders transport of DNA in proximity of cells consequently leading to relatively low transfection. Studying different parameters of gene electrotransfer *in vitro* under the conditions that more closely

resemble the *in vivo* conditions, especially mobility would offer the possibility to obtain more efficient gene transfer *in vivo*.

In order to have more realistic *in vivo* model system, we used previously described 3-D collagen model (52), which we developed for analysis of gene electrotransfer. In the first part of our study we therefore compared gene electrotransfer efficiency on: (i) plated cells, (ii) cells grown on top of collagen layer (which represent the intermediate step between classical cell culture and *in vivo* model system) and (iii) cells embedded in 3-D model. As we expected gene electrotransfer efficiency was substantially higher, when cells were plated as a monolayer culture compared to cells grown on top of collagen layer or cells embedded in 3-D model for all pulsing protocols. The result of our experiments showed that maximum gene electrotransfer efficiency was obtained, when pulses of longer duration were used, where 54% of plated cells, 13% of cells grown on top of collagen layer and 2.5% of cells embedded 3-D model were successfully transfected. The difference in gene electrotransfer efficiency can be mostly explained by the fact that plasmid DNA transport through collagen matrix is relatively slow, especially when cells are embedded in 3-D model. Our results in 3-D model can be compared to the results of *in vivo* experiment, where also relatively low gene electrotransfer (around 2%) was obtained (58).

To understand if the decrease in gene electrotransfer efficiency is not a consequence of reduced cell viability, we tested survival of cells 24 h after electric pulse application for plated cells, cells grown on top of collagen layer and cells embedded in 3-D model. As already shown by others (18), when we increased pulse duration also cell viability was reduced. Indeed, pulse duration should be optimized to obtain sufficient gene electrotransfer efficiency and to avoid irreversible cell damage. Interestingly, when cells were embedded in 3-D model, cell viability was higher when longer pulses were used (8 x 5 ms) compared to plated cells or cells grown on top of collagen layer. We suggest that higher viability in 3-D model is a consequence of smaller DNA concentration in a vicinity of cells, since it was shown by others, that high levels of plasmid DNA can be toxic (59).

Furthermore, *in vivo* DNA is usually delivered to the target cells by means of a local injection (23, 38, 48, 49) and consequently only cells in vicinity of injected site are in close contact with DNA. Thus in the second part of our study we analyzed DNA

mobility in a 3-D model by applying DNA on top or injected it into the 3-D model. We observed that the latter way of application showed higher transfection efficiency compared to the former one. The highest gene electrotransfer efficiency was obtained for both ways of DNA application, when a train of eight pulses with 2 ms duration and *E*= 1.0 kV/cm was used. At those conditions the highest transfection obtained was around 6% when DNA was injected into 3-D model, compared to approximately 4%, when DNA was applied on top of 3-D model. We also observed that more cells were successfully transfected near the injection site (Fig. 6). Cell viability was for both ways of DNA application in 3-D model always similar (data not shown).

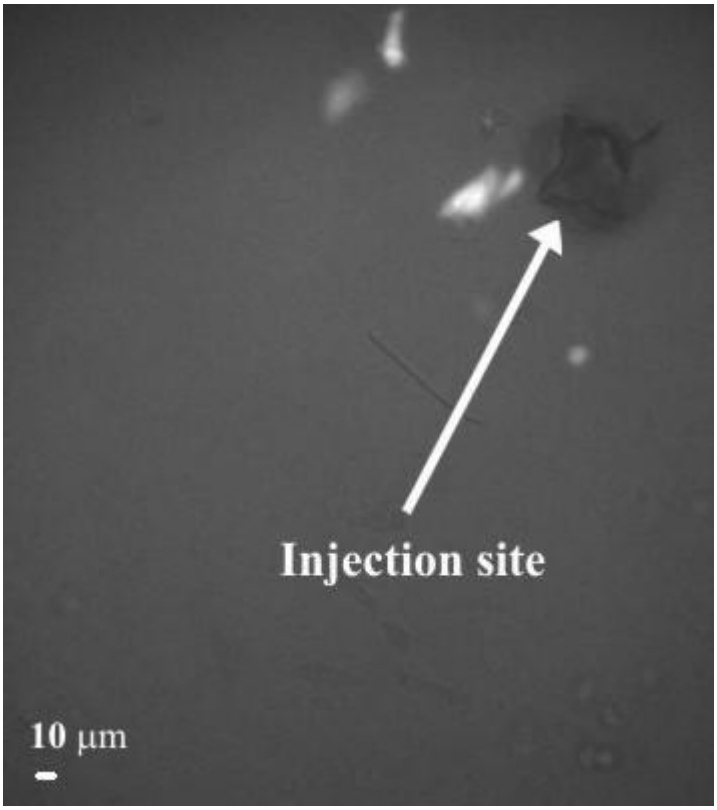

Figure 6: Representative picture, which shows that especially cells in the 3-D model, which were near the injection site were successfully transfected. Eight pulses of 2 ms long, pulse repetition frequency of 1 Hz and *E* = 0.8 kV/cm were applied. Plasmid concentration in electroporation media was 90 μg/ml.

Since it was shown by many *in vivo* (23, 39, 48 - 50, 56) studies that short high-voltage (HV) microsecond pulses in combination with long low-voltage (LV)

millisecond pulses contribute to higher gene electrotransfer efficiency, we in the third part of our study analyzed the influence of different combinations of HV and LV pulses on gene electrotransfer efficiency in 3-D model. We obtained higher gene electrotransfer efficiency when using HV 2 (8 x 200 μs; 0.8 kV/cm; 1 Hz) pulse in combination with LV 2 (1 x 100 ms; 150 V/cm) pulse, compared to using only HV 2 pulse. At those conditions also cell viability was preserved. Our results are in agreement with the *in vitro* study of Kanduser *et al*., where it was suggested that LV pulses are *in vivo* conditions crucial for efficient transfection as they electrophoretically move DNA towards the cells (24).

The main limitation of our 3-D model was very low cell viability for LV pulses with longer durations (more than 200 ms long). We therefore could not analyze the influence of even longer LV pulses on gene electrotransfer efficiency in 3-D model.

In the third part of our study we also analyzed the influence of changing the electric field orientation on gene electrotransfer efficiency in 3-D model. For this purpose we applied: (i) single polarity (SP) and (ii) orthogonal both polarities (OBP) pulses. It was already suggested, that by changing the polarity of the pulses the membrane area that is competent for DNA entry into the cell increases. Our results in 3-D model were consistent with previous results (25, 57) where gene transfection is increased when the electric field orientation between electrical pulses is changed.

Classical 2-D cell cultures do not reproduce the morphology and biochemical features that cells possess in tissue. As alternative, cells grown in more *in vivo* like environment such as collagen gel, offer the possibility to study different parameters of gene electrotransfer. We therefore analyzed mobility of DNA during gene electrotransfer in our 3-D model and pointed out how important it is that DNA is in closer proximity of a cell in order to achieve successful gene electrotransfer. We also analyzed the influence of different combinations of HV and LV pulses and changing the polarity of pulses on gene electrotransfer efficiency in 3-D model.

We showed that our 3-D model can be used to study different mechanisms of gene electrotransfer. By that we could optimize gene delivery *in vivo* and reduce the number of animals used for *in vivo* experiments.

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Poglavje 5

Primerjava elektroporacije in lipofekcije za vnos plazmida pEGFP-N1 v humane mioblastne celice *in vitro*

Comparison of electroporation and lipofection for *in vitro* transfer of plasmid pEGFP-N1 into human myoblasts

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POVZETEK

Eden izmed glavnih ciljev raziskovanja *in vitro* je razvoj učinkovite metode za vnos zdravila. Učinkovit vnos genskega materiala v celice je bistvenega pomena za uporabo v genski terapiji ali DNA imunizaciji. Različne molekule in genski material lahko na različne načine vnašamo v evkariontske celice. Cilj naše študije je bila primerjava učinkovitosti in toksičnosti elektroporacije in lipofekcije. Elektroporacija je metoda, kjer z električnimi pulzi povečamo prepustnost celične membrane, kar nam omogoči vnos DNA v celico. Lipofekcija, je metoda kjer DNA v celico vnašamo z liposomi. Takšna DNA se v celico prenese z endocitozo. Obe metodi imata svoje prednosti in slabosti, kar je odvisno od vrste uporabe (*in vitro*, elektrogenska terapija, utišanje genov). Poznavanje primernih parametrov za učinkovit vnos DNA v celice pri obeh metodah je pomembno, saj tako lahko preučujemo mehanizme vnosa genov in izboljšamo metod v okolju *in vivo*. Trenutno se elektroporacija uporablja tako *in vitro*, kot *in vivo*, ter predstavlja obetavno metodo za gensko transfekcijo in DNA imunizacijo v kliničnem okolju. Lipofekcija pa je bolj primerna za razvijanje strategij genske terapije v okolju *in vitro*, čeprav se uporablja tudi *in vivo.* Plazmid pGFP-N1, ki nosi zapis za zeleni fluorescentni protein, smo vnesli v primarne celice človeških mioblastov z elektroporacijo ali lipofekcijo (Lipofectamine 2000). Najvišja stopnja transfekcije je bila za obe metodi primerljiva, in sicer 40 %. Citotoksičnost metode pa je bila pri elektroporaciji višja (60 % preživelih celic) v primerjavi z lipofekcijo (80 % preživelih celic). Naši rezultati kažejo, da je za *in vitro* vnos DNA v primarne mišične mioblaste zaradi višjega preživetja celic primernejša lipofekcija, obe metodi pa sta učinkoviti, saj lahko dosežemo do 40 % transfekcijo celic.

Ključne besede: elektroporacija, lipofekcija, humani mioblasti, plazmid pEGFP-N1, *in vitro*.

Izjava: Podpisana Saša Haberl izjavljam, da moje samostojno delo v okviru doktorata v tem prispevku obsega analiza uspešnosti *in vitro* vnosa DNA v primarne mišične mioblaste z metodo elektroporacije in določanje preživetja celic.

ABSTRACT

One of the main goals of *in vitro* research is to develop efficient methods for drug delivery. Efficient delivery of genetic material into cells is crucial for application in clinical environment for gene therapy or gene vaccination. Genetic material and foreign molecules can be introduced into eukaryotic cells by various ways. The aim of our study was to compare the efficiency and toxicity of two different methods for transfer of plasmid DNA: electroporation, where electric pulses are used to permeabilize cell membrane, which consequently enables transfer of plasmid DNA; and lipofection where liposomes are used as vehicles for DNA transfer into the cell. Each of these methods has its advantages and disadvantages depending on the type of application (*in vitro*, electrogene therapy, gene silencing). Electroporation is currently promising for clinical application in electrogene therapy (EGT) and for gene vaccination, while lipofection is auspicious for developing strategies for gene therapy mostly *in vitro*. Since each of these methods has its own advantages, it is important to know which parameters determine efficiency of both methods *in vitro* as well to understand the mechanism involved in gene transfer in order to optimize *in vivo* applications of gene electrotransfer or lipofection. Plasmid pEGFP-N1 coding for green fluorescent protein was transfected into primary human myoblasts either with lipofection (Lipofectamine 2000) or applying high-voltage pulses. The highest rate of transfection achieved by electroporation and lipofection was around 40%. However, cytotoxicity of electroporation was higher (60% viable cells) compared to lipofection (around 80% viable cells). Our results showed that more suitable method for *in vitro* transfection studies on myoblasts is lipofection, due to higher viability of the cells.

Keywords: electroporation, lipoflection, human myoblasts, plasmid pEGFP-N1, *in vitro*.

1 INTRODUCTION

Gene therapy can represent an elegant way to cure serious diseases at both the somatic cell and germline cell level (1). It is believed to be the therapy of the 21st century as it aims to eradicate cause rather than symptoms of diseases (2). Mayor factor limiting successful gene therapy is the difficulty of expressing new copies of therapeutic genes adequately within target cells and tissues (1). Consequently, a lot of attention and interest is directed towards designing efficacious and safe transfection vectors (2).

A very large number of techniques for the transfection of mammalian tissue culture cells have been described (3). In the present study we compared the efficiency and cytotoxicity of two non-viral methods for *in vitro* cell transfection: lipofection and electroporation.

We applied a cationic liposome reagent Lipofectamine 2000 (Invitrogen) for lipofection, which provides high transfection efficiency and high levels of transgene expression in various mammalian cell types *in vitro* (3). Cationic liposomes are composed of cationic lipids containing a hydrophobic domain and one or more positively charged functional groups in their head-group region (2). For successful transfection, a nucleic acid, which carries a net negative charge under normal physiological conditions, must come into contact with a cell membrane that also carries a net negative charge. Lipofectamine 2000 forms a complex with nucleic acid molecules, thus allowing them to overcome the electrostatic repulsion (3). Current view of lipofection pathways include: a) endocytotic internalization of the lipid-DNA complex (lipoplex), b) release of endosomally trapped lipoplex to cell cytoplasm, and c) transport of the released DNA to the nucleus followed by its expression (2).

Lipofection efficiency depends on several basic parameters such as cell density, amount of DNA and Lipofectamine 2000, DNA:Lipofectamine 2000 ratio, dilution time of Lipofectamine 2000 before adding DNA, time of DNA:Lipofectamine 2000 complex formation, and cell culture conditions.

In this study we compared lipofection with another promising gene delivery method that is based on delivering of electric pulses to the cells - electroporation.

Gene electrotransfer is caused by temporary increase in cell membrane permeability (electroporation), nevertheless the exact molecular mechanisms of DNA transport across the membrane are still not known. Different mechanisms that explain DNA entry to the cytosol have been suggested (4).

One of the key parameters for successful electroporation and increased membrane permeability is the induced transmembrane voltage. This voltage is generated by an external electric field due to the difference in the electric properties of the membrane, cytoplasm and the external medium. The transmembrane voltage (when above certain critical level) induces strong electric field inside the cell membrane, which is crucial for destabilization of membrane and formation of structural changes (pores) inside the lipid bilayer, which consequently increases membrane permeability for ions and molecules. However, it was further shown that the process of gene electrotransfer is more complex than simple DNA diffusion through membrane pores. It was shown, that the crucial step in DNA delivery is the interaction (adsorption) of a DNA molecule with the cell membrane, which is followed by DNA translocation (4). The movement of DNA across the cell membrane is a very slow and a post pulse event (5).

The role of different parameters that effect transfection efficiency is well known. The field strength must be high enough to trigger membrane electropermeabilization and DNA must be present during the field application on cells. The level of expression is dependent on the amount of added DNA. Pulse duration must be long enough to obtain an efficient level of expression. Limitations in the field strength and pulse duration are due to damaging effect of too high/too long pulses on the cell integrity (5).

In our experiments, we transfected primary muscle cell cultures - myoblasts with 4.7 kb long plasmid pEGFP-N1 coding for green fluorescent protein.

2 MATERIALS & METHODS

2.1. Preparation of myoblast cultures

Primary myoblast cultures were prepared as described previously (6). Briefly, satellite cells were prepared from muscle tissue routinely discarded at orthopedic operations on patients without muscular disease. The muscle tissue was cleaned of adhering connective tissue, cut into small pieces, and trypsinized to release muscle satellite cells. Cells were grown at clonal density in 100 mm petri dishes in advanced minimum essential medium (aMEM) supplemented with 10% fetal bovine serum (FBS) at saturated humidity in a mixture of 5% CO_2 and air at 37°C. Confluent myoblast cultures were trypsinized before myoblast fusion. Cells were plated in 12-well (lipofection) and 24-well (electroporation) dishes and grown to attain adequate confluence; 90-95% for lipofection, 10-20% for electroporation, because close contacts between cells may act as physical barriers that limit the diffusion of plasmid DNA when electrical pulses are applied (7).

2.2. Lipofection

Twenty-four hours before transfection, growth medium was replaced with the medium without antibiotics and antimycotics.

DNA-Lipofectamine 2000 complexes were prepared as followed: for each transfection sample we diluted the adequate volume (2, 3 and 4 μL) of Lipofectamine 2000 stock solution in 100 μL Opti-MEM without serum. After 5 min we combined the Lipofectamine 2000 (Invitrogen, GB) solution with previously prepared DNA solution (1.6 μg DNA was diluted in 100 μL Opti-MEM without serum).

The solution was let to stand at room temperature (22ºC) for 20 min to allow the Lipofectamine 2000-DNA complexes (lipoplexes) to form.

Meanwhile, medium without antibiotics and antimycotics was changed to Opti-MEM without serum. After 20 min we added 200 μL of transfection complex to each well, containing cells and medium.

The cells were then incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. After 6 h of lipofection, we changed medium for MEM, supplemented with 10% fetal bovine serum. After 24 h we stained the cells with Hoechst stain 33342.

2.3. Electroporation

Culture growth medium was replaced with potassium buffer (KH_2PO_4/K_2HPO_4, 10 mM solution, containing 1 mM $MgCl_2$, 250 mM sucrose) and DNA in concentration 40 μg/mL. Cells were incubated for 3 min, and then electric pulses were delivered with Jouan GHT 1287B generator. A pair of parallel wire electrodes was used with the distance between the electrodes being 4 mm.

Two different pulsing protocols were used: a) 8 pulses in duration of 1 ms (1 Hz), with three amplitudes: E = 0.7 kV/cm, E = 0.8 kV/cm and E = 0.9 kV/cm and in protocol b) 8 pulses in duration of 2 ms (1Hz), also with three amplitudes: E = 0.7 kV/cm, E = 0.8 kV/cm and E = 0.9 kV/cm. Pulses were applied at room temperature (T=22°C).

After pulse delivery, cells were incubated for 5 min at 37°C, and then the potassium buffer solution was removed and replaced with aMEM, supplemented with 10% FBS. The cells were then incubated for 24 h at 37°C in a humidified incubator with 5% CO_2.

2.4. Transfection efficiency evaluation

The efficiency of transfection is normally presented as the percentage of cells translating and accumulating the protein of interest in the total cell population.

In our case transfection efficiency was determined by fluorescent microscopy with excitation light at 445 nm (suitable to detect GFP) and 355 nm (suitable to detect Hoechst stain) generated with a monochromator system; emission was detected at 488 nm (GFP; successfully transfected cells) and 465 nm (Hoechst stain; total cell population).

We manually counted the cells and the relative transfection efficiency was determined by the ratio between the number of GFP-positive cells and the total cell number determined by Hoechst staining (lipofection) and number of counted cells under phase contrast microscopy (electroporation). Three independent experiments were performed for method and results are presented as a mean values±standard deviation.

2.5. Cytotoxity determination

Cytotoxicity of lipofection was determined with Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Germany).

Survival of the cells after electroporation was obtained from phase contrast images as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample (no electrical pulses were delivered to the control sample).

2.6. Statistical analyses

Results are expressed as mean±SD. To test for differences among groups one-way ANOVA was used, followed by Bonferroni's *post hoc* test for multiple comparisons. Data were analyzed using SPSS 15.0 for Windows (SPSS, Chicago, IL, USA).

3 RESULTS

In this study we compared two different methods for *in vitro* transfection of human myoblasts: lipofection and electrotransfection. We determined the efficiency of transfection and cytotoxicity for both methods.

3.1. Efficiency of DNA transfection

After 24 h incubation time, 40.9±4.2% (n=3) was the highest rate of successfully transfected cells using lipofection method. The structure of lipofection complex, that was the most efficient, was, when using: 1.6 µg of plasmid and 4.0 µL of Lipofectamine 2000 stock solution. Reduced rates of transfection were observed at lower Lipofectamine 2000 concentrations (Fig. 1).

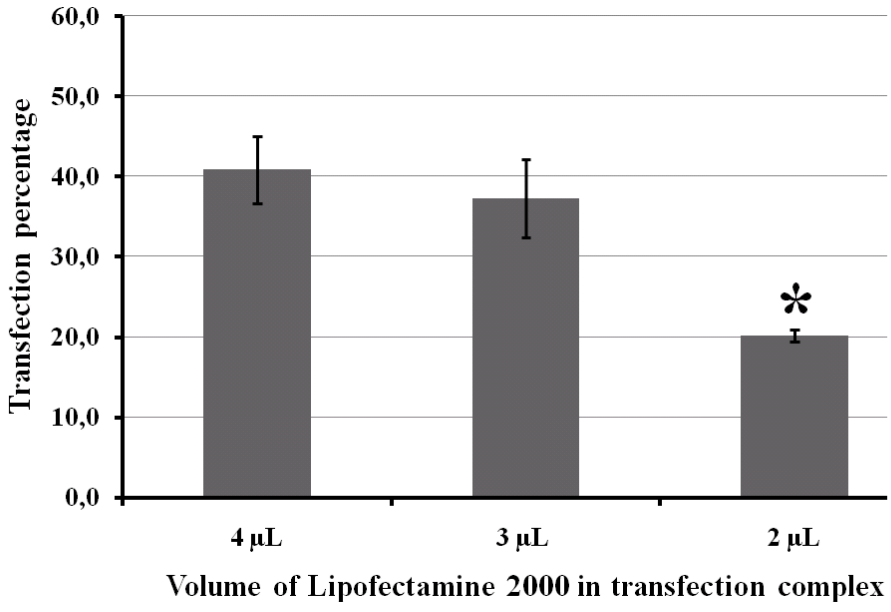

Fig. 1: The effect of different Lipofectamine 2000 volume parts in 200 µl of lipofection complex on transfection. The quantity of plasmid is constant (1.6 µg).

For gene electrotransfer, the highest rate of successfully transfected cells with electroporation was 41.4±15.8% (n=3), obtained by applying 8 × 2 ms pulses: with 0.8 kV/cm applied electric field. Transfection was overall higher for 8 × 2 ms pulses compared to 8 × 1ms (Fig. 2).

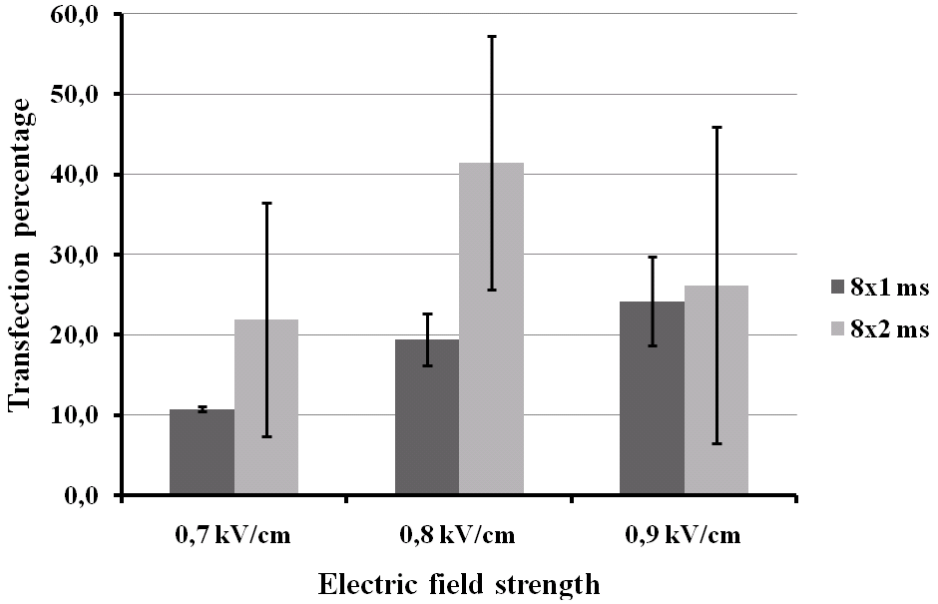

Fig. 2: The effect of different electric field strengths and duration of electric pulses on transfection.

3.2. Cytotoxicity of DNA transfection

Lipofection cytotoxicity was much lower in comparison with cytotoxicity observed by electroporation, ranging between 8.8±10.2% and 21.8±8.1% (n=3) (Fig. 3). In lipoplex with combination 1.6 µg of DNA and 4.0 µL of Lipofectamine 2000 stock solution, where the highest transfection ratio was obtained, the cytotoxicity was 21.8±8.1% (n=3).

Cytotoxicity was higher by electroporation. Under conditions where the highest transfection efficiency was achieved (electric field strength 0.8 kV/cm, pulses delivered 8 times in duration of 2 ms), the cytotoxicity was 63.9±9.2% (n=3). Otherwise, the

range of cytotoxicity by electroporation was between 25.9±18.6% and 68.7±8.1% (n=3) (Fig. 4).

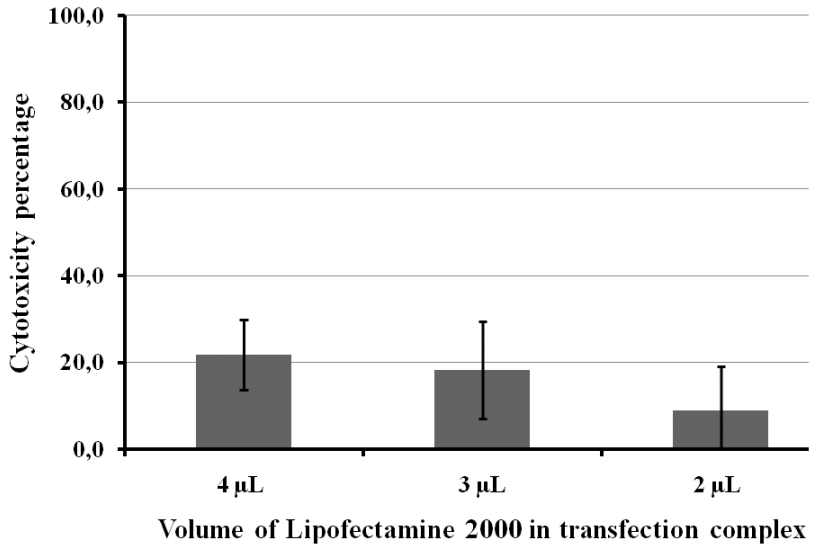

Fig. 3: The effect of different Lipofectamine 2000 volume parts in 200 µl of lipofection complex on cytotoxicity (percentage of dead cells). The quantity of plasmid was constant (1.6 µg).

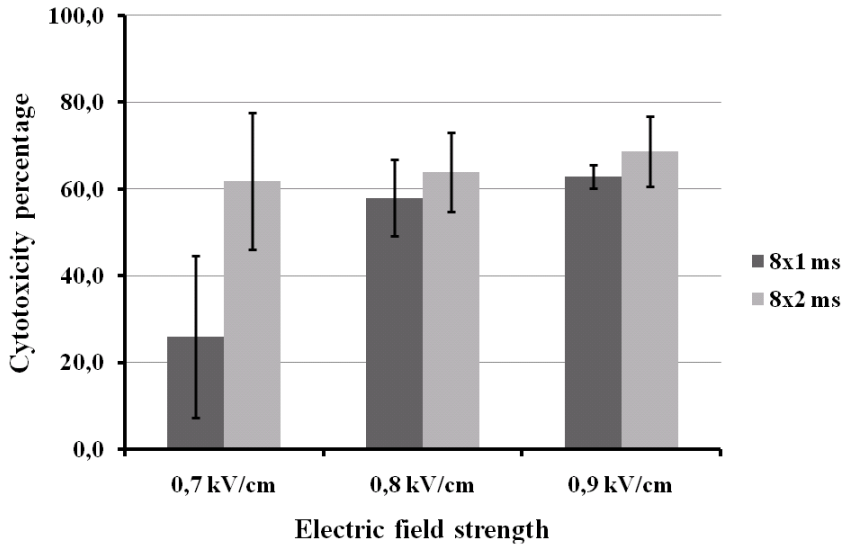

Fig. 4: The effect of different electric field strengths and pulse durations of electric pulses on citotoxicity of gene electrotransfer.

4 DISCUSSION & CONCLUSIONS

Our results demonstrated that efficient transfection of human myoblasts with DNA *in vitro* can be obtained both with electrotransfection as well as with lipofection.

The highest transfection ratios by both methods were quite similar (40.9% with lipofection versus 41.4% with electroporation) however the main difference was in cytotoxicity of both methods.

Less than 40% of cells were viable after the electroporation under conditions that give comparable transfection results with lipofection. On the other hand, by lipofection, almost 80% of myoblast were viable after the treatment. These results show that the more convenient method for *in vitro* transfection studies on myoblasts is lipofection.

We are confident, that with further experiments and with the use of another lipofection reagent, higher transfection efficiencies can be achieved.

Also for some applications *in vivo* such as gene therapy and gene vaccination, electroporation has advantage over lipofection due to simplicity of protocol and fact that no additional chemicals are used. Electric pulses delivery is already regularly used for localized drug delivery in the treatment of cutaneous and subcutaneous solid tumors by electrochemotherapy (8). Because recent technological developments made DNA electrotransfer more and more efficient and safer, this gene therapy approach is now ready to reach the clinical stage (9). Therefore it is very important that we continue with the research and optimization of electroporation as transfection method in *in vitro* and *in vivo* conditions.

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ZAKLJUČNA RAZPRAVA

Genska elektrotransfekcija je metoda, ki z uporabo električnih pulzov omogoča vnos DNA v celico. Prednost te metode je predvsem varen in preprost način vnosa genskega materiala, zato metoda predstavlja najbolj obetavno alternativo virusni transfekciji na področju genske terapije. Prvič je bila genska elektrotransfekcija opisana v 80-ih letih, vendar mehanizmi vnosa DNA v celico z dovajanjem električnih pulzov niso še popolnoma razjasnjeni. Znano je, da je prvi in nujni pogoj za uspešnost metode vezava DNA na elektropermeabilizirano celično membrano. Sledi prenos DNA čez membrano, migracija skozi citoplazmo in vstop v jedro celice. Nekateri avtorji poudarjajo vlogo dvovalentnih kationov kot pomembnih dejavnikov pri zmanjšanju elektrostatske odbojne sile med negativno nabito DNA in negativno nabito celično membrano. S tem je vezava DNA na membrano celice učinkovitejša, kar lahko vodi v izboljšanje transfekcije. Najpogosteje uporabljeni dvovalentni kationi v elektroporacijskem mediju so Mg^{2+} ioni, ki zato predstavljajo pomemben del raziskovanja mehanizmov genske elektrotransfekcije in možnosti izboljšanja metode.

Vpliv Mg^{2+} ionov v elektroporacijskem mediju na različne korake genske elektrotransfekcije

V doktorski nalogi je prikazan vpliv povečane koncentracije Mg^{2+} ionov v elektroporacijskem mediju na izboljšano elektropermeabilizacijo membrane, ki je prvi nujni pogoj za uspešno transfekcijo genov. Za indikator elektropermeabiliziranosti membrane smo uporabili majhno molekulo propidijev jodid, ki ne prehaja v celico skozi neprepustno membrano.

Učinkovitejša elektropermeabiliziranost membrane pa ni vodila k učinkovitejši genski elektrotransfekciji, kot smo predvidevali. Pri 50 mM koncentraciji Mg^{2+} ionov v elektroporacijskem mediju je bila uspešno transfecirana le četrtina celic v primerjavi z 1 mM koncentracijo. Ker bi lahko bila znižana učinkovitost genske elektrotransfekcije posledica slabšega preživetja celic, smo analizirali vpliv Mg^{2+} ionov na preživetje celic po dovajanju električnih pulzov. Z višanjem koncentracije Mg^{2+} ionov smo pridobili višje preživetje elektroporiranih celic, kar je v skladu s študijami *in vivo,* ki so pokazale, da Mg^{2+} ioni pripomorejo k hitrejšemu okrevanju porušene elektrolitske homeostaze celice.

V nadaljevanju smo se posvetili analizi vezave DNA na celično membrano v prisotnosti najnižje (1 mM) ali najvišje (50 mM) koncentracije Mg^{2+} v elektroporacijskem mediju, saj bi lahko večja količina Mg^{2+} ionov vezala DNA na membrano s tako afiniteto, da DNA ne bi mogla prehajati skoznjo. Molekula DNA je rahlo negativno nabita molekula in se v električnem polju giba v smeri anode. Tako se s fluorescentnim barvilom TOTO-1 obarvana DNA po dovajanju električnih pulzov v eni smeri nakopiči na membrani celice, ki se nahaja na strani katode. Po prvi seriji električnih pulzov v eni smeri je sledilo dovajanje električnih pulzov enakih parametrov v nasprotni smeri, s čimer smo želeli odcepiti že vezano DNA na membrani. Po dovajanju drugega vlaka pulzov v nasprotni smeri smo opazili padec fluorescence na celični membrani pri 1 mM koncentraciji Mg^{2+} ionov, medtem ko tega ni bilo opaziti pri 50 mM koncentraciji Mg^{2+} ionov. Naši rezultati kažejo, da višja koncentracija Mg^{2+} veže DNA na membrano s takšno intenziteto, da DNA ostane vezana na membrani in ne more prehajati skoznjo. Genska elektrotransfekcija je zelo kompleksen proces in Mg^{2+} ioni bi lahko poleg interakcije DNA s celično membrano vplivali tudi na druge korake transfekcije. V literaturi je zaslediti, da ti ioni lahko povečajo aktivnost znotrajceličnih DNaz, ki razgrajujejo v celico vneseno DNA. Z uporabo Zn^{2+} kot inhibitorja DNaz so pokazali, da se uspešnost genske elektrotransfekcije poveča. V naši raziskovalni nalogi smo zato uporabili Zn^{2+} pri različnih koncentracijah Mg^{2+} v elektroporacijskem mediju in opazovali vpliv na gensko elektrotransfekcijo. Pri dodajanju Zn^{2+} takoj po dovajanju električnih pulzov nismo opazili izboljšanja učinkovitosti genske elektrotransfekcije, zato sklepamo, da Mg^{2+} ioni ne vplivajo na stabilnost DNA v citoplazmi celice, temveč je njihov vpliv pomemben predvsem pri vezavi DNA na celično membrano.

3-D model *in vitro* za študijo genske elektrotransfekcije

Številne predklinične študije na živalskih modelih so poskušale optimizirati gensko elektrotransfekcijo, še vedno pa predstavlja največji problem učinkovitost metode *in vivo*. Celice, gojene v kulturi *in vitro*, ne izražajo enakih lastnosti kot celice v tkivu. Prav tako tudi zunajcelično ogrodje s svojo gostoto vpliva na zmanjšano gibljivost DNA in s tem na učinkovitost genske elektrotransfekcije. Zato je potrebna optimizacija metode v pogojih, ki bi zadovoljivo posnemali okolje *in vivo*.

Razvili smo preprost tridimenzionalni (3-D) model celic gojenih v kolagenu, ki nam je omogočal analizo vpliva različnih parametrov na učinkovitost genske elektrotransfekcije. V tako gojene celice smo z dovajanjem električnih pulzov uspešno vnesli DNA, naši rezultati so bili primerljivi z rezultati, pridobljenimi v okolju *in vivo*.

Analizirali smo vpliv vnosa različnih koncentracij DNA in vpliv različno trajajočih pulzov na učinkovitost genske elektrotransfekcije celic, gojenih v 3-D kolagenskem modelu. Pokazali smo, da s poviševanjem koncentracije DNA v elektroporacijskem mediju narašča tudi uspešnost transfekcije. Tudi trajanje pulzov je pomemben dejavnik, ki vpliva na vnos DNA v celico, daljši električni pulzi namreč pripomorejo k boljši elektromobilnosti DNA, kar poveča uspešnost metode. Naši rezultati kažejo, da z uporabo daljših pulzov narašča tudi uspešnost genske elektrotransfekcije, vendar le do določene mere, saj predolgi električni pulzi povzročijo nepopravljive poškodbe celične membrane, zato takšna celica ne preživi. Tako je nujno potrebna optimizacija protokola za doseganje visoke transfekcije in hkrati dobrega preživetja celic.

V nadaljnjih preučevanjih mehanizmov genske elektrotransfekcije v 3-D celičnem modelu smo preučevali vpliv mobilnosti in elektromobilnosti DNA. V tkivu kompleksna struktura zunajceličnega ogrodja zmanjša mobilnost in elektromobilnost DNA, posledično je genska elektrotransfekcija manj učinkovita.

Zmanjšano mobilnost DNA smo prikazali s primerjavo uspešnosti genske elektrotransfekcije na pritrjenih celicah *in vitro*, na celicah, gojenih na kolagenski podlagi, in na celicah, gojenih v 3-D celičnem modelu. Genska elektrotransfekcija celic v 3-D modelu je bila močno znižana v primerjavi s pritrjenimi celicami v kulturi *in vitro*, kar lahko pripišemo močno zmanjšani mobilnosti DNA v kolagenski strukturi, ki ponazarja zunajcelično ogrodje v okolju *in vivo*.

V poskusih *in vivo* z injiciranjem DNA v tkivo dosežejo boljši stik DNA s celicami, saj jo tako aplicirajo direktno na mesto dovajanja električnih pulzov. Prav tako smo tudi v 3-D modelu prikazali izboljšano učinkovitost genske elektrotransfekcije pri injiciranju DNA v kolagensko strukturo.

Na mobilnost DNA v tkivu lahko vplivamo z dovajanjem kombinacije visokonapetostnih in nizkonapetostnih pulzov. Kratek visokonapetostni električni pulz omogoči permeabiliziranost membrane, medtem ko daljši nizkonapetostni pulz prispeva

k elektroforezi negativno nabite DNA. V doktorski nalogi je predstavljen vpliv različnega trajanja pulzov in različnih kombinacij visokonapetostnih in nizkonapetostnih pulzov na gensko elektrotransfekcijo v 3-D modelu. Pokazali smo, da s primerno izbiro kombinacije visokonapetostnega in nizkonapetostnega pulza izboljšamo učinkovitost transfekcije.

K povečani učinkovitost metode prispeva tudi obračanje smeri električnih pulzov. Z dovajanjem električnih pulzov v vseh smereh omogočimo povečano prepustnost večje površine membrane in tako večji vnos DNA v celico. Naši rezultati so pokazali, da se genska elektrotransfekcija v 3-D modelu v primerjavi z dovajanjem pulzov samo v eni smeri poveča, če dovajamo električne pulze v vseh smereh.

Primerjava elektroporacije in lipofekcije za vnos DNA v humane mioblastne celice *in vitro*

Glavno ciljno tkivo genske elektrotransfekcije za vnos terapevtskih genov je mišično tkivo. Mišične celice so dobro prekrvavljene in izražaje produktov terapevtskih genov je dolgotrajno, učinki terapije pa so vidni tako lokalno kot tudi sistemsko. Za optimizacijo transfekcije v kliničnih aplikacijah je zelo pomembna analiza tudi na primarnih humanih mišičnih celicah *in vitro*.

V doktorski nalogi je predstavljena primerjava dveh najpogostejših načinov transfekcije mišičnih celic – lipofekcija in genska elektrotransfekcija. Na primarni celični liniji človeških mioblastov smo optimizirali parametre za uspešno gensko elektrotransfekcijo in dobro preživetje celic. Pokazali smo, da je uspešnost lipofekcije in genske elektrotransfekcije primerljiva, medtem ko je citotoksičnost lipofekcije manjša. Zato lipofekcija predstavlja boljšo metodo za študijo transfekcije primarnih celic človeških mioblastov *in vitro*, vendar v okolju *in vivo* prednjači genska elektrotransfekcija zaradi večje učinkovitosti, preprostosti metode in dejstva, da z njo v tkivo ne vnašamo dodatnih kemikalij.

Predstavljene raziskave pripomorejo k razlagi mehanizmov genske elektrotransfekcije in hkrati omogočajo izboljšanje učinkovitosti metode v kliničnih aplikacijah genske terapije, kjer je še vedno problem majhna učinkovitost.

SKLEPI

Vpliv Mg^{2+} ionov v elektroporacijskem mediju na različne korake genske elektrotransfekcije:

- Pokazali smo sistematično analizo vpliva Mg^{2+} ionov na posamezne korake genske elektrotransfekcije. Dokazali smo, da je po dovajanju električnih pulzov prepustnost celične membrane za majhne molekule (elektropermeabilizacija membrane) povečana v prisotnosti večje koncentracije Mg^{2+} ionov, medtem ko je uspešnost genske elektrotransfekcije znižana. Uspešna elektropermeabilizacija celične membrane je nujni, vendar ne zadostni dejavnik za uspešno gensko elektrotransfekcijo tudi ob prisotnosti večjih koncentracij Mg^{2+} ionov;
- s študijo genske elektrotransfekcije v prisotnosti različnih koncentracij Mg^{2+} ionov smo dokazali, da ti vplivajo predvsem na interakcijo DNA s celično membrano. Pokazali smo, da je ob višji koncentraciji Mg^{2+} ionov intenziteta vezave DNA na membrani celice dovolj visoka, da DNA ne more prehajati skozi membrano, kar povzroči manj uspešno gensko elektrotransfekcijo;
- z inhibicijo znotrajceličnih DNaz v prisotnosti različne koncentracije Mg^{2+} ionov smo pokazali, da razgradnja transfecirane DNA v citoplazmi ni odločujoč dejavnik za manj uspešno gensko elektrotransfekcijo.

Izdelava 3-D modela *in vitro* za študijo elektropermeabilizacije in genske elektrotransfekcije:

- Zgradili smo 3-D model tkiva *in vitro*, kjer so celice gojene med kolagenskimi vlakni, in kot tak predstavlja vmesni model med okoljema *in vitro* in *in vivo*. Pokazali smo uspešen vnos majhnih molekul in kot prvi tudi vnos DNA v tovrsten 3-D model tkiva *in vitro* z dovajanjem električnih pulzov. Določili smo optimalno koncentracijo DNA in parametre električnih pulzov za doseganje uspešne genske elektrotransfekcije v 3-D modelu.

Vpliv zmanjšane mobilnosti in elektromobilnosti na gensko elektrotransfekcijo v 3-D modelu *in vitro*:

- Sistematično smo prikazali uporabnost zgoraj omenjenega 3-D modela *in vitro* za napoved elektroporacijskega protokola za maksimalen vnos genov v kliničnem

okolju *in vivo*, kjer je doseganje želene učinkovitosti genske elektrotransfekcije še vedno problem;

- Z različnim načinom aplikacije DNA v 3-D model ter z gojenjem celic na površini 3-D modela ali v njegovi notranjosti smo pokazali znižano mobilnost DNA zaradi gostih, polimeriziranih kolagenskih vlaken, ter s tem zmanjšano uspešnost genske elektrotransfekcije;
- pokazali smo, da primerna kombinacija visokonapetostnih in nizkonapetostnih električnih pulzov vpliva na boljšo elektromobilnost DNA med kolagenskimi vlakni v 3-D modelu in s tem na višjo uspešnost genske elektrotransfekcije.

Primerjava elektroporacije in lipofekcije za vnos DNA v humane mioblastne celice *in vitro*:

- Pokazali smo uspešen vnos DNA z dovajanjem električnih pulzov v primarne humane celice mišičnih mioblastov in določili primerne parametre električnih pulzov s katerimi dosežemo dovolj visoko preživetje celic in hkrati optimalen vnos DNA;
- s primerjavo vnosa DNA z dovajanjem električnih pulzov in z lipofekcijo v primarne humane celice mišičnih mioblastov smo pokazali, da je uspešnost obeh metod transfekcije DNA primerljiva in hkrati dokazali, da je najprimernejša metoda za vnos genov v primarne humane celice mišičnih mioblastov *in vitro* lipofekcija, saj v primerjavi z gensko elektrotransfekcijo omogoča višje preživetje celic.

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