Optimization of electroporation parameters for delivery of small molecules into primary human myotubes

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Optimizacija parametrov eletroporacije za vnos majhnih molekul v primarne misične cevčice

Abstract. *Za vnos genov obstajajo različne metode in virusna transfekcija je trenutno metoda z najvišjo stopnjo vnosa. Vendar pa zaradi tveganja uporabe virusnih vektorjev v človeškem organizmu, ki lahko povzroči hude stranske efekte, obstaja potreba po alternativnih metodah. V zadnjih desetletjih je genska transfekcija z elektroporacijo postala ena najbolj obetavnih metod za nevirusni vnos genskega materiala za gensko terapijo in za DNA vakcinacijo. Lokalno dovedeni električni pulzi povečajo prepustnost membrane in omogočijo prenos plazmidne DNA (pDNA) ali kratkih RNA v celice. V zadnjem času se je genska elektrofekcija izkazala tudi kot zelo primerna metoda za genski vnos pri DNA vakcinaciji za zdravljenje raka, AIDS in drugih infekcijskih bolezni. Čeprav lahko vnašamo gene v različna tkiva, pa se je skeletna mišica pokazala kot idealno tarčno tkivo za vnos genov, saj je mišično tkivo enostavno dostopno, predstavlja velik delež telesa, ter omogoča dolgotrajno izražanje genov. Zaradi kompleksnosti in vivo raziskav pa je potrebno veliko postopkov najprej izvesti v bolj definiranem in vitro okolju. Humani mioblasti diferenciirani v in vitro pogojih v mišične cevčice predstavljajo zanimiv model skeletne mišice za optimizacijo vnosa malih molekul in plazmidne DNA. V tej študiji smo izvedli optimizacijo vnosa malih molekul z elektroporacijo in analizo preživetja na modelu humanih primarnih mišičnih cevčic.*

1 Introduction

In the last decades, in vivo gene transfer using electroporation (gene electrotransfer) has been established as an efficient non-viral method of delivery for gene therapies [1-3].

Importantly, in the last decade DNA electrotransfer was show to be a very successful approach for DNA vaccination strategies where electric pulses provide additional stimuli to immune system. Electroporation based DNA vaccination has been successfully applied for cancer treatment, AIDS and various infectious diseases [2]. Specifically, skeletal muscle was found to be an ideal target tissue for electrotransfer since high efficiency of transfer could be obtained. Skeletal muscle represents large portion of body mass that is relatively easily accessible and in addition has several metabolic and physiological properties that make it a suitable target for novel biomedical applications [1-4].

In parallel, over the past two decades also short RNA based therapeutics emerged. Electroporation based delivery of short RNA (shRNA, siRNAs, miRNA) is already widely used for gene silencing and other therapeutic strategies [5-7].

The mechanisms of molecular delivery by electroporation are still being explored; however, in general the method is based on locally delivered electric pulses that can transiently increase membrane permeability and thus enable transfer of small molecules (drugs, fluorophores, short RNAs) and large molecules like plasmid DNA (pDNA) into the cells. Several steps that are crucial for successful gene electrotransfer were so far identified: i) electropermeabilization of the cell membrane, ii) contact of pDNA with the cell membrane (DNA-membrane complex), iii) translocation of pDNA across the membrane, iv) transfer of pDNA to and into the nucleus and v) gene expression [2,3,7-9]. However, there is no complete description of the mechanism of gene electrotransfer on a molecular level. Also, despite the routine use of the electrotransfer in biotechnological applications the efficiency in tissue still represent one of major limitations for use in clinical setting.

Electroporation is a threshold phenomenon where permeabilization can be achieved only above some critical transmembrane potential for the specific target cells. However, for the specific application of electroporation the critical transmembrane voltage U_c is not known and depends on the chosen type of cell, the duration of electric pulses and the number of pulses. Therefore, optimization of pulse parameters has to be performed for every new target cells or target tissue.

One additional crucial parameter that determines efficiency of electrotransfer or gene silencing is viability of cells after electroporation. Electroporation induces pores in the membrane, which temporarily disrupts cell homeostasis due to leakage of ions and molecules out and into the cells. If the electric pulses are too strong the cell membrane cannot reseal leading

to cell death (irreversible electroporation). Therefore, in all optimization protocols it is also important to optimize for optimal delivery and viability depending on the type of the final application.

Only few studies were done so far on differentiated primary myotubes as an in vitro model of skeletal muscle tissue, particularly of human origin. Due to species specificity of muscle cell, primary cultures of human muscle cells are currently the only model from which results can be extrapolated to in vivo conditions in humans.

Based on potentials of short RNA therapies we have optimized electroporation conditions for electroporation-based delivery of small molecules into primary human myotubes cultured in vitro. This can be used for therapeutic purposes or alternatively, silencing of a specific gene can enable us to study selectively different signaling pathways relevant for various therapeutic approaches and studies of muscle regeneration.

2 Materials & Methods

2.1 Cultured human myoblasts and myotubes

Muscle cultures were prepared as described in detail before [10]. Briefly, myoblast cultures were prepared from muscle tissue routinely discarded at orthopedic operations. Muscle tissue was cleaned of connective and adipose tissue, cut to small pieces, and trypsinized at 37°C to release muscle satellite cells. Isolated cells were grown in 100-mm petri dishes (BD Falcon, Franklin Lakes, NJ) in growth medium AdvancedMEM supplemented with 10% (vol/vol) FBS, 0.3% (vol/vol) fungizone, and 0.15% (vol/vol) gentamicin (all obtained from Invitrogen, Paisley, UK) at 37°C in 5% CO2-enriched atmosphere at saturation humidity. Purity of myoblast cultures was increased using the CD56 MACS system. CD56+ cells were transferred to new cell culture flasks, and were grown under the same conditions as the primary cultures for two to three more passages, when they were used for experiments.

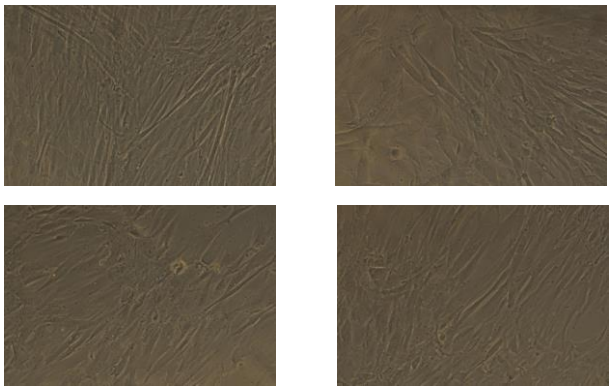

Figure 1: Differentiated myotubes after 6th day differentiation.

Differentiation of myoblasts into myotubes was perfromed by using AdvancedMEM supplemented with 2% (vol/vol) FBS. The differentiation into myotubes was observed by bright field microscopy, and well-differentiated cells were observed 6th day after initiation of differentiation (Fig.1). Electroporation was performed on 9th day of differentiation.

Experiments were carried out on cells plated in Lab-Tek II 4-Chamered slides (Thermo Fisher Scientific, MA; USA). The study was approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (permit No: 71/05/12).

2.2 Plasmid DNA

Plasmid pGFP-N1 (Clontech Laboratories Inc., Mountain Viw, CA, USA) encoding green fluorescent protein (GFP, excitation 488 nm, emission 507 nm) 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

The permeabilization threshold and the extent of permeabilization was obtained by electroporation of cells in RPMI media without FBS (electroporation buffer) containing 0.15 mM propidium iodide (PI) (Sigma Aldrich), a short term membrane impermeable fluorescent dye.

Electric pulses were generated by BetaTech pulse generator. A pair of parallel wire electrodes with 9.5 mm distance between them (d) was used. Electrodes were positioned on the bottom edges of the sample chamber in order to expose all cells to electric pulses. The electric field (E) is homogeneous and can be calculated by the formula $E = U / d$, where U denotes applied voltage and d the electrode distance.

All pulsing protocols consisted of 8 consecutive square pulses of frequency 1 Hz. The amplitudes of the applied electric fields (E) were used depending on the pulse length. For 8 × 2 ms pulses the amplitudes of voltages were 100V, 200V, 500V, 600V, 700V 800V with applied fields E = [0.105, 0.21, 0.52, 0.63, 0.73 and 0.84] kV/cm. For 8 × 5 ms pulses voltages were 100V, 200V, 300V, 400V, 500V, 600V with E = [0.105, 0.21, 0.315, 0.42, 0.52 0.63] kV/cm. Pulses with 100 V and 200 V amplitudes were used only to determine the electroporation threshold and were not used in viability experiments. Cells in the control samples were by the same procedure but were not electroporated – no pulses were applied.

5 min after pulse delivery electroporation buffer was removed, cells were washed and finally basic advanced-MEM media with 2% FBS was added. Phase contrast and fluorescent images were taken at 20× objective magnification for each sample.

2.4 Cell viability

Electroporation for viability analysis was performed similarly as for electropermeabilization (2.3) except that immediately after electroporation 25%vol. FBS was added as in previous studies of electrotransfection or silencing [2,7,8,10]. After 10 min incubation at 37°C 800 µl advanced-MEM media was added. Cells were allowed to grow for 24h at 37°C in a humidified 5% CO_2 atmosphere. Cells in the control sample (K0) were treated with the same protocol, but without exposure to electric pulses (E = 0 kV/cm).

Viability of myotubes was determined 24h after electroporation and was analyzed by fluorescent spectroscopy (Tekan Infinite, Tekan, Germany). Cells were lysed with a 0.04% sodium dodecyl sulfate solution .and then buffer containing 50 mM TRIS-HCl, 100 mM NaCl (pH = 8.25) and 5 µg/ml Hoechst 33342 stain (Thermo Fisher Scientific) was added to each well. The number of cells was determined using Hoechst staining [illegible] solution .and th (Tecan, Männedorf, Switzerland).

The percentage of viable cells (% Viability) in a given sample was determined as the ratio between the fluorescence intensity of the treated sample (*FLs*) and the fluorescence intensity of the negative control FL_0: % %Viability = 100 × FLs/FL_0. Two independent experiments were performed on the cells of two different donors. The results were pooled together and are presented as mean ± standard error.

2.5 Electrotransfection

Plasmid DNA pEGFP-N_1 coding for GFP (green fluorescent protein) was used to test gene electrotransfer. Cells were electroporated in RPMI media with 40 µg/ml pEGFP using a train of 8 × 2 ms pulses with 400V and 600 V. Immediately after electroporation 25%vol. FBS was added. After 10 min incubation at 37° 800µl alpha MEM media was added. Bright field and fluorescent images of GFP expression were taken 24h after electroporation.

3 Results & Discussion

3.1 Optimization of electropermeabilization of primary human myotubes

Electropermeabilization experiments were performed to determine the permeabilization threshold of primary human myotubes exposed to a wider range of electric field strengths delivered for 8 × 2 ms pulses and 8 × 5 ms with repetition frequency 1 Hz. The duration and number of pulses were selected based on our previous optimizations experiments on primary human myoblasts and myotubes. The permeabilization threshold E_c for 8 × 2 ms and 8 × 5 ms was below 100 V (0.105 kV/cm), where for longer pulses the threshold was expectedly little lower. Since our primary focus was to establish a protocol for efficient delivery of small molecules together with relatively preserved viability with PI as a model molecule, we have tested the range of voltages that resulted in electropermeabilization and preserved viability. For 8 × 2 ms almost all cells were permeabilized already at 700 V while for 8 × 5 ms 100% permeabilized cells were observed at 600 V. With electric field strength 0.4 kV/cm and above, all cells were permeabilized (Fig. 2).

Consistently, with increasing electric field strength and increasing number of permeabilized cells, maximal fluorescent values increased, indicating that more PI entered the cells. With higher voltages, the fluorescence increased despite all cells were electroporated. Based on the permeabilization results, pulses with U = 500-800 V for 8 × 2 ms and U = 300 – 600 V for 8 × 5 ms and higher were used for further viability experiments.

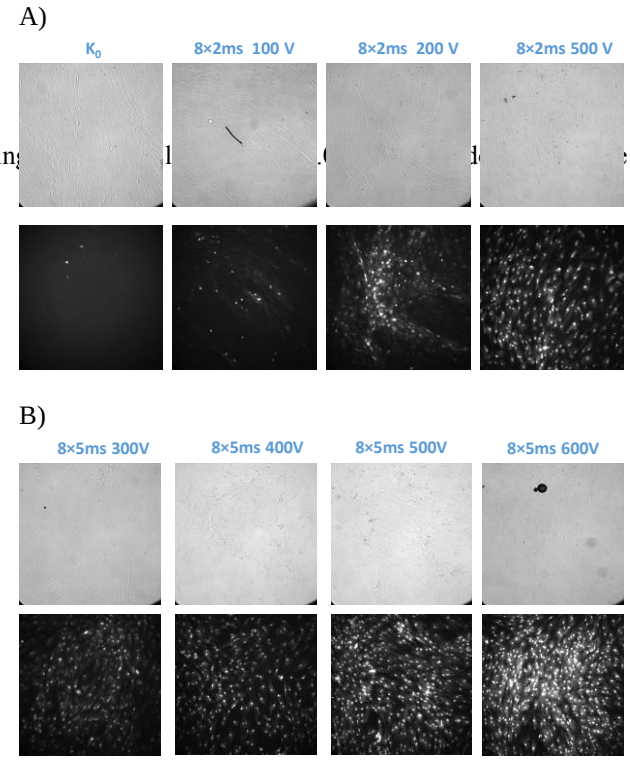

Figure 2: Effect of increasing electric field strength on permeabilization of primary human myotubes. Cells were electroporated in the presence of 0.15 mM PI using a train of A) 8 × 2 ms and B) 8 × 5 ms for increasing applied voltage. Cells in the control sample K_0 were not electroporated (U = 0 V). Bright field and fluorescent images were taken 5-10 minutes after electroporation.

3.2 Viability

Viability of myotubes was determined 24h after electroporation. The percentage of viable cells (%Viability) in a given sample was determined as the ratio between the number of viable cells and the number of viable cells in the negative control as determined by fluorescent spectroscopy and Hoechst staining (Figure 3).

As it can be seen, there was substantial variability between the two donors, which is in agreement with previous data. In general some decrease in viability due to delivered pulses was obtained (80%) however, more

experiments would have to be performed in order to obtain more accurate data.

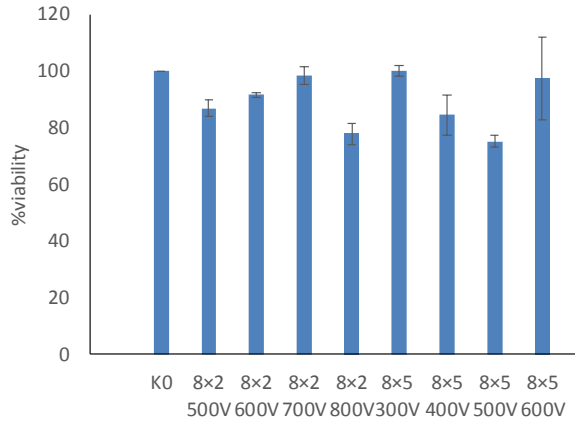

Figure 3: Effect of electroporation parameters on viability of primary human myotubes. The percentage of viable cells (%Viability) is shown as the ratio between the fluorescence intensity of the treated sample and the fluorescence intensity of the negative control K0. Cells were electroporated using 8 × 2 ms and 8 × 5 ms for increasing applied voltage. Cells in the control sample K0 were not electroporated (U = 0 V).

Finally, we have tested possibility of electrotransfer delivery of plasmid DNA in primary human myotubes. Based on our previous studies and electropermeabilization experiments we have chosen 8 × 2 ms pulses with 400V and 600V.

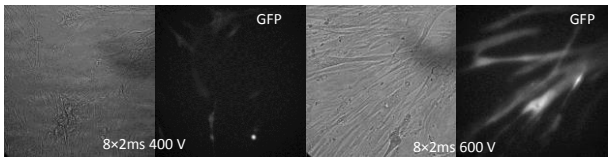

Figure 4: Electrotransfection of primary human myotubes. Cells were electroporated in the presence of 40 µg/ml pEGFP using a train of 8 × 2 ms pulses with 400V (left) and 600 V (right). Bright field and fluorescent images of GFP expression were taken 24h after electroporation.

We have obtained efficient electrotransfer of pEGFP plasmid into primary human myotubes using 8 × 2 ms with U = 600 V, while 400 V resulted only in few transfected cells as shown in Fig. 4. This is in agreement with our previous studies where we have shown that efficient electrotransfer is achieved above the electropermeabilization threshold and at the voltages that enable very efficient electropermeabilization close to 100% with viabilities above 75% [2].

4 Conclusions

We present results of in vitro optimization of the electroporation protocol for the introduction of small molecules and plasmid DNA into cultured primary human myotubes. By varying electric field strength and duration of the pulses, we have established optimal pulsing protocol with regard to electropermeabilization using small molecule PI on the one hand and preserved viability on the other. Altogether 8 × 2 ms with applied electric field strength between 0.5 to 0.6 kV/cm and 8 × 5 ms electric pulses with applied electric fields 0.3 kV/cm to 0.4 kV/cm should be most optimal for delivery of small molecules and short RNA for silencing experiments. As a proof of concept, we have tested that the pulses optimized for delivery of small molecules could also be efficiently used for electrotransfer into differentiated myotubes and successful GFP expression was achieved.

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