Optimization of electric pulse parameters for permeabilization and viability of primary human chondrocytes in isotonic buffer for cell electrofusion

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Abstract*. Cell fusion plays an important role in tissue regeneration. For biotechnological applications we can induce cell fusion by electroporation. Electroporation brings the cell membrane in so called fusogene state. When we put two fusogene membranes in close contact, cell fusion can take place. Electric pulse parameters must be optimized in order to obtain cell membrane permeabilization and to preserve cell viability. In the present study we tested different pulse numbers and durations: 8×100 µs, 4×200 µs and 8×2 ms and electric field amplitudes ranging from 0.5 to 3 kV/cm. Optimized electric pulse parameters for primary human chondrocytes obtained from one donor used in this study range from 1.25 kV/cm to 1.6 kV/cm depending on the pulse durations. Higher pulse amplitudes are needed for microsecond pulses. With optimized pulses we obtained efficient permeabilization and more than 80% of viable cells. The obtained parameters will be used for cell electrofusion of primary human chondrocytes and mesenchymal stem/stromal cells. Mesenchymal stem cells are used in regenerative medicine and cell hybrids obtained by electrofusion between chondrocyte and mesenchymal stem cells hold great potential for regeneration of cartilage lesions.*

1 Introduction

Cell fusion plays an important role in tissue development, homeostasis and regeneration. In some infectious diseases caused by enveloped viruses [1] or by bacteria [2], the infection results in the host cell fusion and creation of large multinucleated syncytia. According to some authors, cell fusion is a driving force in cancer progression and metastatic spreading [3]. In healthy organism, cell fusion is a highly regulated process required for muscle and placental development or for tissue remodelling. Cell fusion is an important mechanism of tissue regenerating ability. Mesenchymal stem/stromal cells (MSCs) are involved in tissue repair and regrowth and hold great potential for regenerative medicine [4]. One of the mechanisms for their regenerative capacity is cell fusion.

Cell fusion is also used in biotechnological applications for cell manipulation. Hybrid cells are obtained with biological, chemical or physical methods of cell fusion and are mainly used for cell vaccination [5], [6] and for monoclonal antibody production. However, the mechanisms of cell fusion are poorly understood [6]. Cell fusion is a complex phenomenon in which several steps can be distinguished. Cells need to overcome fusion preventing mechanisms and the membranes of fusion partners need to come in close contact. The cell membranes of the fusion partners needs to reach a special, so called fusogene state [5], [6].

Fusogene state of the cell membrane can be induced by the expression of different enveloped viral proteins in the cell membrane [7] or by chemical agents such as polyethylene glycol (PEG) [8]. The current limitation of biological and chemical methods used for hybrid cell production is a relatively scarce number of hybrid cells. Cell electrofusion is yet another, physical method of cell fusion which currently provides a higher number of hybrid cells [9], [10]. The method is based on electroporation and requires external application of high voltage (kV range), very short (micro to millisecond) electric pulses. The external electric field induces transmembrane potential and pore formation in the membrane lipid bilayer, so called cell membrane electroporation or electropermeabilization. When electroporated cell membranes are in close contact, cell fusion takes place [11], [12]. The efficacy of cell electrofusion depends on the method used for cell contact formation [9], electric pulse parameters [10], electroporation buffer and the cell line [9]. Electrofusion is a suitable method of cell fusion for a wide range of different cell types.

The aim of the present study was to determine cell membrane permeabilization and long-term cell viability. Both parameters are required starting point for electrofusion experiments. We have optimised the electroporation parameters for one of the fusion partners, primary human chondrocytes. We tested different electric pulse parameters in order to identify the optimal electrofusion protocol that can also be used

for electroporation of mesenchymal stem/stromal cells (MSCs). Hybrids between primary human chondrocytes and mesenchymal stem cells are expected to poses regenerative properties and can be used for regeneration of cartilage lesions.

2 Materials and methods

2.1 Primary chondrocyte isolation

Primary human chondrocytes were obtained from tissue biopsy from donors undergoing routine clinical examination. The donors signed informed consent to participate in the study. Isolated tissue was stored in DMEM (Dubecos modified minimal essential media (Gibco) supplemented with 10% foetal bovine serum (FBS), L-glutamine, and antibiotics. Chondrocytes were isolated as previously described in [13].

2.2 Electroporation

Cells were cultured in low glucose DMEM supplemented with 10% foetal bovine serum (FBS), L-glutamine, and antibiotics in T75 culture flask. Cells were seeded at the concentration of $0{,}15\times10^5$ cells/ml. On the day of experiment cells were washed with isoosmolar potassium phosphate buffer – KPB (10mM K_2HPO_4/KH_2PO_4, 1mM MgCl2) with 250 mM sucrose and osmolarity 262mOsmol/kg. Cells were trypsinized with trypsin/EDTA for 10 minutes at 37°C. Action of trypsin was deactivated by addition of complete low glucose DMEM. Cells were centrifuged at 300×g for 5 minutes. Supernatant was decanted and cell pellet was resuspended in isoosmolar KPB. For electroporation we used 100 µL of cell suspension in concentration 10^6 cells/ml. Electroporation was performed in electroporation cuvettes with incorporated plate aluminium electrodes (WWR), electrode distance 2 mm. Electric pulses were delivered by electroporator ElectroCell 15 (Leroy Biotech).

For determination of cell membrane permeabilization, we used cell membrane impermeant dye propidium iodide at concentration 15 µM and excitation emission 535/617 nm. Propidium iodide was added to electroporation buffer and cells were left undisturbed for five minutes after electroporation. After 5 minutes 1 ml of complete DMEM was added and images were captured under inverted fluorescence microscope (Evos, Thermo Fisher Scientific). For each parameter we obtained brightfield and fluorescence images. Images were overlaid using image processing software Image J. At least 700 cells were counted and the percentage of permeabilised cells was determined.

For cell survival cells were electroporated in KPB, maintained at 37ºC for 10 minutes to allow cell membrane resealing and plated in 24 well plate (WP). Cell survival was monitored by observation of morphological changes observed two or five days after electroporation. Images were captured under 10× or 20× objective magnifications.

Oxidative stress was measured by fluorescence ROS assay kit Carboxyl-H_2DCFDA (5µM) according to manufacturer instructions. Ten minutes after electroporation cells were placed in a 24 well plate and incubated with the fluorescent dye at room temperature for 5 minutes. Stained cells were transferred to a 96 well plate and fluorescence was measured in microplate reader (Tecan) at 488/522 nm excitation/emission at different time points: 0, 2min, 20 min and 30 minutes. The first measurement was performed 5 minutes after the addition of the dye.

3 Results and discussion

3.1 Permeabilization and oxidative stress

To determine the permeabilization of human primary chondrocytes in isotonic KPB we applied a train of 8 electric pulses with the duration of 100 µs and the repetition frequency of 1 Hz (8×100 µs) at different pulse amplitudes. The percentage of permeabilised cells was determined by manual cell counting of bright field and red fluorescence images. Above 90% of cell membrane permeabilization was reached at pulse amplitude 2 kV/cm (Figure 1).

In further experiments we tested cell membrane permeabilization for different numbers and durations of pulses. For 8×100 µs pulses we used pulse amplitude 2 kV/cm and at the same amplitude we also tested 4×200 µs pulses. Besides we tested longer pulses (2 ms) with pulse amplitude 1.25 kV/cm. The amplitudes of 2 ms pulses were selected on the basis of cell survival results (described later). At all selected amplitudes we obtained more than 95% of cell membrane permeabilization (Figure 2).

Further we tested the level of oxidative stress of primary human chondrocytes exposed to selected electric pulse parameters. The results demonstrate that initial increase in oxidative stress caused by electroporation decreases with time. The time constant of oxidative stress decrease corresponds to cell membrane resealing which is completed thirty minutes after electroporation. No differences were observed among electric pulse parameters in the pulse amplitude range from 0.5 to 2 kV/cm. Oxidative stress caused by electroporation was not affected by different pulse amplitudes (Figure 3 A) or durations (Figure 3 B).

3.2 Cell viability

In order to obtain all the required information for the selection of appropriate electric pulses for cell electrofusion of primary human chondrocytes we determined cell viability for selected electric pulse parameters. Cell viability was determined by morphologic observation of the treated cells two and five days after electroporation. In the figure 4 we

present long term cell viability of primary human chondrocytes exposed to no electric pulses and selected electric pulse parameters. For shorter 8×100 µs electric pulses cell morphology was not affected up to 1.6 kV/cm (Figure 4A). For longer pulse duration (2 ms) cell morphology was not affected up to 1 kV/cm. At electric pulse amplitude 1.5 kV/cm cell morphology was affected and the number of cells surviving was slightly lower (Figure 4B).

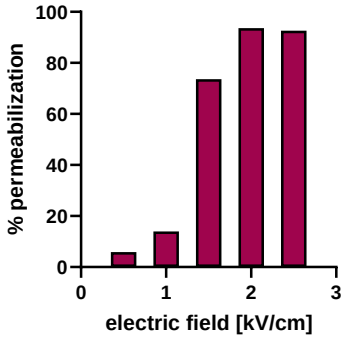
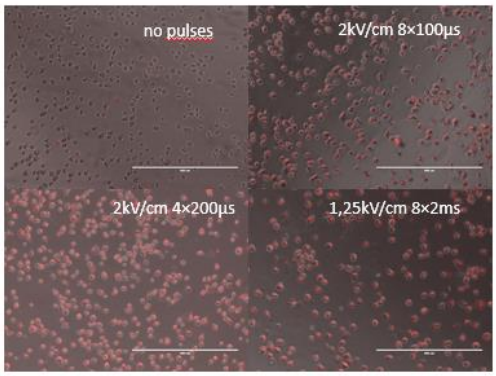


Figure 1: Permeabilization of primary human chondrocytes (donor 3) determined by uptake of fluorescent dye propidium iodide in control (no electric pulses were applied) and electroporated cells exposed to electric pulse 8×100 µs applied at repetition frequency 1 Hz.

A

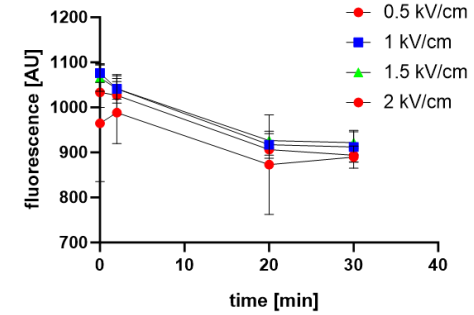


B

primary human chondrocytes	
parameters	permeabilization
no pulses	1,5%
4×200µs 400V	99,0%
8×100µs 400V	99,1%
8×2ms 250V	97,6%

Figure 2: Permeabilization of primary chondrocytes with different electric pulse parameters. Composed images showing overlay of brightfield and fluorescence image (PI) demonstrating over 95% of permeabilised cells. Images were captured at 10× objective magnification. Scale bar 200 µM (A). The percentage of electroporated cells in control treatment (cells not exposed to electric pulses), and cells exposed to 8×100 µs electric pulses 2 kV/cm, 4×200 µs pulses 2 kV/cm and 8×2 ms pulses 1.25 kV/cm (B).

A



B

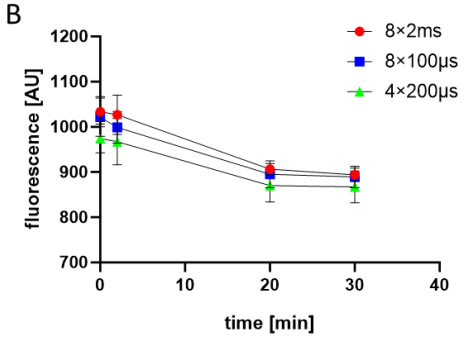


Figure 3: Oxidative stress caused by electroporation of primary human chondrocytes by fluorescent dye Carboxyl-H_2DCFDA. Cells were stained for 5 minutes 10 minutes after electroporation. A fluorescence measurement was performed in 4 time points: 0 corresponds to the first measurement, immediately after placing the sample in the microplate reader. The measurement was repeated at 2, 20 and 30 minutes. Electric pulse parameters were 8×100 µs at different pulse amplitudes (A). Different pulse durations the pulse amplitude 2 kV/cm for 8×100 µs and 4×200 µs and 1.25 kV/cm for 8×2 ms pulses (B). Measurements were performed in 4 technical replicates.

A

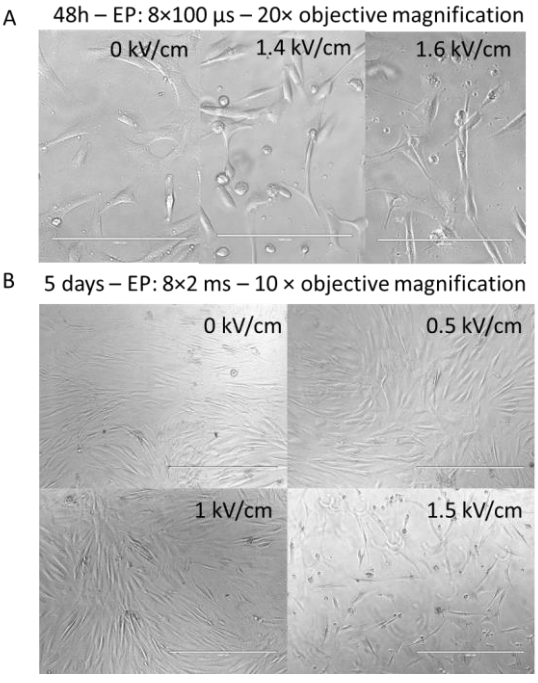


Figure 4: Cell morphology and cell survival of primary human chondrocytes (donor 3) exposed to different electric pulses.

Viability was determined two or five days after electroporation. Electric pulses 8×100 µs at 1.4 kV/cm and 1.6 kV/cm 48h after electroporation were compared to control, cells not exposed to electric pulses 0 kV/cm. Images were captured at 20× objective magnification (A). For longer electric pulses (8×2 ms) cell morphology was observed five days after electroporation. Electric field amplitudes were 0.5 kV/cm, 1 kV/cm, 1.5 kV/cm and 2 kV/cm. Images were captured at 10× objective magnification (B). At 2 kV/cm 8×2 ms pulses cell morphology and survival were drastically affected (images not shown).

4 Conclusion

The obtained results provide a suitable range of electric pulse parameters that enable cell membrane permeabilization and preserve cell viability of primary human chondrocytes. The experiments were performed on primary cells obtained from a single donor. We can expect some interindividual differences between different donors in response to electric field treatment, therefore more experiments need to be done before we can select the optimal electric pulses for cell electrofusion. As our goal is to produce cell hybrids between chondrocytes and MSC, we need to optimize the electroporation parameters for MSC as well. The wide range of electric pulse parameters that can be used for electroporation of primary human chondrocytes are a good starting point. Measurements of oxidative stress related to electroporation demonstrate that the selected pulses allow the treated cell to recover from the stress in the time course of cell membrane resealing.

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