Optimization of electroporation of primary human chondrocytes in hypoosmolar electroporation buffer

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Abstract. *Tissue regeneration is a highly regulated physiological process that aims at maintaining tissue homeostasis. In osteoarthritis this process becomes dysregulated due to a reduced capacity of joint cartilage resident cells (chondrocytes) in maintaining tissue homeostasis. Mesenchymal stem/stromal cells (MSCs) are generally the main players in tissue regeneration, with important mechanism of their tissue regenerating ability being cell fusion.*

Primary human chondrocytes from three donors were used. Electroporation was carried out in hypotonic buffer and pulses (8×100 µs, 1 Hz) of varying amplitudes were applied. Our results show that the amplitudes needed to achieve reversible electroporation were at 1,0 kV/cm. Cell viability remained high even at 1,8 kV/cm, though the metabolic activity and overall cell number dipped significantly in amplitudes above 1,4 kV/cm. Based on these data we can conclude that pulses from 1,0 to 1,4 kV/cm enable successful electroporation of chondrocytes, not significantly affecting their viability.

In our study we have observed the successful electropermeabilization and cell viability above 70% of primary human chondrocytes after electroporation. Establishing this sort of groundwork will give base for further research and an eventual establishment of successful fusion cells between primary human chondrocytes and other fusion partner cells.

1 Introduction

Tissue regeneration is a highly regulated physiological process that is operational during the entire lifespan and aims both, at maintaining tissue homeostasis, as well as restoring functionality of injured tissues and organs. An important aspect of tissue regeneration is cell fusion [1]. This physiological process has mostly been studied in the setting of embryonic development, for example in myogenesis, bone formation and placentation [2]. The importance of cell fusion is reflected in the fact that its dysregulation can cause various diseases, e.g. osteoporosis, multiple sclerosis and preeclampsia [3]. However, the process of cell fusion remains only scarcely understood, particularly in terms of the mechanisms and factors (e.g. fusogenic proteins and others) that induce, mediate and terminate plasma membranes merging between various cell types.

High energetic and mechanistical barriers need to be overcome to achieve successful cell fusion, which makes it a rare spontaneous event [4]. Several studies show that hematopoietic stem and MSCs are able to restore degenerated tissue by adopting the phenotype of various cell types via cell fusion [3]. Cell fusion is then followed by dedifferentiation and formation of new hybrid pluripotent stem cells. These cells are then capable of differentiation and can aid in regeneration of targeted tissue [3], [5]. MSCs are also characterised by anti-inflammatory (immunomodulatory) potential, low allogeneic immunogenicity and high homing capacity [6]. Therefore, they can induce and regulate tissue regeneration via both, paracrine activity and direct differentiation into specific tissue cells.

Autologous and allogeneic MSCs present the key components of advanced therapy medicinal products (ATMPs). The development of ATMPs is in its steep ascending phase, showing good therapeutic results in the treatment of degenerative pathologies, such as joint degeneration. Osteoarthritis is the most common form of joint degeneration. In 2020, 595 million people worldwide suffered from osteoarthritis, representing as much as 7.6% of the world population, and a 132.2% increase compared to 1990. Projections indicate a further 74.9% increase in the incidence of knee and a 78.6% increase in hip osteoarthritis by 2050 [7].

The two key features of MSCs are their anti-inflammatory (immunomodulatory) activity [8] and capacity to directly, or via cell fusion, differentiate into various cell types of connective tissues, i.e. bone, cartilage, tendon, muscle, adipose and others [9]. While immunomodulatory function of MSCs and their differentiation capacities in vitro are well understood, their regenerative properties, based on cell fusion with target tissue cells, are still largely unknown.

Our study aims at optimizing electroporation parameters for electrofusion of primary human chondrocytes, setting up basis for achieving successful cell fusion between chondrocytes and primary human MSCs, which would allow development of models for

studying cell fusion *in-vitro* and eventual development of hybrid cell based ATMPs.

2 Materials and methods

2.1 Isolation of primary human chondrocytes

With approval of the National Medical Ethics Committee (Code 0120-268/2020/3), we isolated primary human chondrocytes from lateral and medial condyles of patients undergoing total knee replacement surgery at University Medical Centre Maribor. The donors signed an informed consent form to participate in the study. We included samples from three donors in this study. Isolated tissue was stored in complete cell media (high glucose DMEM (Dulbecco's modified minimal essential media (Biowest)), supplemented with 10% foetal bovine serum (FBS), L-glutamine, and antibiotic/antimycotic) and transported to our laboratory. Cartilage tissue pieces were transferred into sterile bijoux tubes filled with sterile phosphate buffer solution (PBS) and weighed. Samples were transferred to a 15 mL falcon tube filled with complete cell media, supplemented with 1 mg/mL collagenase type II. They were incubated at 37°C for 24h with shaking. The cell suspension was passed through a sterile 40 µm strainer and washed with complete cell media. Cells were then cultured in 10 mL of low glucose DMEM supplemented with 10% FBS, L-glutamine and antibiotic/antimycotic and passaged at 80-90% confluence. The number of cells was counted at each passage and cells were plated at the concentration of 2000 cells/cm^2.

2.2 Electroporation

Cells were washed with isoosmolar potassium phosphate buffer (10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$) with 250 mM sucrose and osmolarity 260 mOsm/L. Cells were detached using trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) at 37°C for 7 minutes. The activity of Trypsin-EDTA was deactivated using complete culture media. Cells were centrifuged at 300× g for 10 minutes and resuspended in isoosmolar potassium phosphate buffer at concentration of 10^6 cells/mL. Cell suspension was transferred to 1,5 mL plastic centrifuge tubes, 100 µL per tube, centrifuged at 300× *g* for 10 minutes. Three minutes before electroporation, isoosmolar buffer was carefully aspirated and replaced by 100 µL hypoosmolar potassium phosphate buffer (10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$ with 75 mM sucrose and osmolarity 93 mOsm/L). It was previously shown that exposing cells to the hypoosmolar buffer for up to 5 minutes causes cells to swell and in turn significantly increases the percentage of successfully permeabilized cells while not significantly affecting their viability [10], [11].

Cell suspension was transferred into 2 mm gap electroporation cuvettes and pulses (8×100 µs 1 Hz) of varying electric field strengths were applied using the ELECTROcell B15 HV+LV electroporator (Leroy Biotech). The electric field strengths that were applied ranged from 0,6 kV/cm to 1,8 kV/cm, depending on the nature of the experiment.

2.3 Cell permeabilization assay

To determine the level of cell membrane permeabilization, we added 15 µM of cell impermeant dye propidium iodide (ex/em 535/617 nm) to the cell suspension prior to electroporation. Upon electroporation, cells were left in the buffer for 5 minutes to ensure membrane resealing. The number of successfully electroporated cells was determined using the Attune NxT (Invitrogen) flow cytometer.

2.4 Cell viability assays

To determine cell viability, we seeded the electroporated cells in 12 well-plates and cultured them in 1 mL of cell media. After 72h the supernatant was transferred into 1,5 mL plastic centrifuge tubes. The cells that remained attached were washed with PBS and detached using trypsin-EDTA as described above. Upon inactivation of trypsin-EDTA with cell media, the cell suspension was transferred into separate 1,5 mL centrifuge tubes and centrifuged at 300× *g* for 10 minutes. Then it was combined with the supernatant from before and again centrifuged at 300× *g* for 10 minutes. Cells were then washed twice with PBS. A small aliquot of cells was transferred to a separate 1,5 mL centrifuge tube and exposed to the temperature of 75°C for 2 minutes. These cells were then cooled on ice for 1 minute, combined with the control group in 1:1 ratio and served as a positive staining control. Cells were stained with eBioscience™ Fixable Viability Dye eFluor™ 780 (Invitrogen) (ex/em 633/780) following the manufacturer recommended protocol. They were put on ice protected from light for 30 minutes. Cells were washed twice with cold PBS, resuspended in approximately 200 µL of PBS and their viability was determined using the Attune NxT flow cytometer. We gated the events to gate out the cellular debris and captured a minimum of 10,000 cells in each of the tested samples. We used the positive control sample to gate two distinct populations of live and dead cells and used these gates to determine the viability of our control sample.

We measured the cell metabolic activity as an alternative mean of determining cell viability. Immediately upon electroporation, cells were seeded into sterile black 96 well-plates with clear bottom at the concentration of about 5,000 cells per well. 68h after electroporation, we added the PrestoBlue metabolic assay reagent. 72h after electroporation, cell morphology was observed using an inverted microscope (EVOS, Thermo Fischer) at 4x objective magnification and fluorescence was measured at 590 nm in a microplate reader (Spark, Tecan Group Ltd.).

3 Results and discussion

3.1 Cell permeabilization

To determine cell membrane permeabilization of primary human chondrocytes, we applied 8×100 µs electric pulses at the repetition frequency of 1 Hz at differing pulse amplitudes, ranging from 0,6 kV/cm to 1,2 kV/cm. We used cells obtained from three different donors that we internally coded as donors RPD1, RPD3 and RPD5. The percentage of successfully permeabilized cells was

determined using flow cytometry. As shown in Figure 1, the increase of successfully permeabilized cells was already statistically significant at pulse amplitudes of 0,8 kV/cm, though there was some observable variance between cells of different donors. Cells from donors RPD1 and RPD5 were highly permeabilized at those amplitudes (86% and 90% respectively), while permeabilization remained low for cells from donor RPD3 (30%). Cells from all of the three donors were successfully permeabilized at pulse amplitudes above 1,0 kV/cm, prompting us to pick this value as our threshold as it guaranteed permeabilization in a wider array of tested cells.

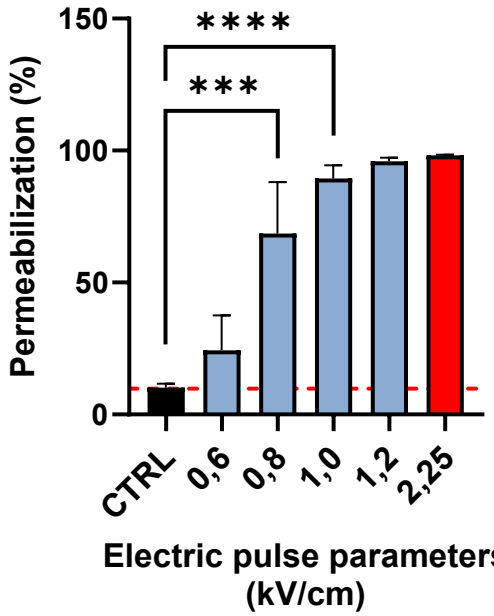

Figure 1. Permeabilization of primary human chondrocytes. *Cells were exposed to different electric pulses. Uptake of cell impermeant dye propidium iodide (15 µM) was determined using flow cytometry. The 2,25 kV/cm data point (red) marks the positive control group. Data are means ± SEM of three independent experiments. One-way ANOVA (post hoc Tukey); *** and **** denote p < 0.001 and p < 0.0001.*

3.2 Cell viability

To determine cell viability of primary human chondrocytes, we applied 8×100 µs electric pulses at the repetition frequency of 1 Hz at different pulse amplitudes, ranging from 1,0 kV/cm to 1,8 kV/cm. We used cells from the same three donors as for the cell permeabilization assay. Cell survival remained high even at the highest tested electric pulse amplitudes, where cell survival dropped to 90% compared to control group, indicating that primary human chondrocytes are durable cells, viability of which is not negatively affected by strong electric pulses (Figure 2). We picked the electric field strength of 1,8 kV/cm as our highest tested value as we anticipate the other fusion partner cells to be reversibly electroporated at strengths below 1,8 kV/cm.

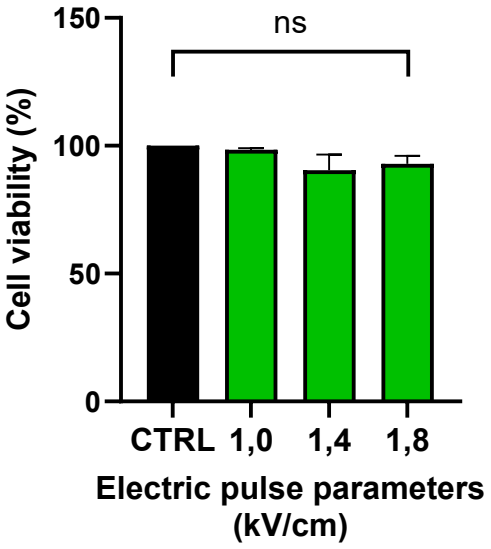

Figure 2. Cell viability of primary human chondrocytes 72h post electroporation. *Cells were exposed to different electric pulse parameters, incubated for 72h and stained with viability dye. Cell viability was determined using flow cytometry. Data are means ± SEM of three independent experiments. One-way ANOVA (post hoc Tukey); ns denotes p > 0.05.*

Additionally, we also measured the metabolic activity and observed cells of donors RPD3 and RPD5 under the inverted microscope for any potential morphological differences. Cells were again exposed to electric pulses of 1 kV/cm, 1,4 kV/cm and 1,8 kV/cm and seeded in clear bottom black 96 well plates. Then, they were cultured at 37°C, 5% CO_2 for 68h before adding the PrestoBlue metabolic assay reagent. The metabolic activity of cells was measured 72h after electroporation when we also took images of cells. In Figure 3 we present the metabolic activity of primary human chondrocytes 72h after being exposed to a train of 8×100 µs pulses in hypoosmolar electroporation buffer.

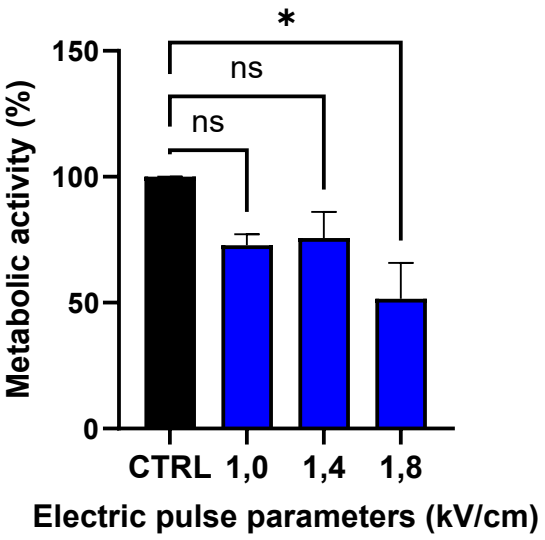

Figure 3. Metabolic activity of primary human chondrocytes 72h post electroporation. *Cells were exposed to different electric pulse amplitudes. Cells were incubated in*

*clear bottom black 96 well plates for 72h. Cell fluorescence was measured at 590 nm. Data are means ± SEM of two independent experiments. One-way ANOVA (post hoc Tukey); ns and * denote $p > 0.05$ and $p < 0.05$ respectively.*

In Figure 4, we present the representative images of cells from donor RPD5 72h after electroporation, along with the count of overall numbers of the cells from both donors. Interestingly, both the cell count, and the overall metabolic activity appear to be lower in cells that were exposed to higher electric field strengths, while their morphology remains largely unaffected. The metabolic activity of the tested cells dropped to 73% and 76% compared to the control group in the cells that were exposed to the electric pulses of 1,0 kV/cm and 1,4 kV/cm respectively. The metabolic activity was significantly lower (52%) for cells exposed to electric pulses of 1,8 kV/cm. Similarly, the overall cell count dropped to 70% and 73% for cells treated with pulses of 1,0 kV/cm and 1,4 kV/cm respectively and was significantly lower (45%) in the group of cells that were exposed to the pulses of 1,8 kV/cm.

a

	CTRL	1 kV/cm	1.4 kV/cm	1.8 kV/cm
%	100,00	70,25	73,75	45,42
SEM	3,49	1,13	15,58	4,44

b

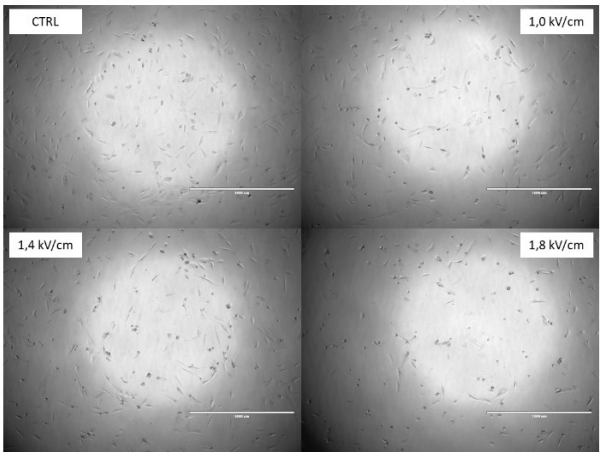

Figure 4. Cell morphology and survival of human chondrocytes exposed to electric pulses. *Cells were exposed to different electric pulse parameters and were compared to the control group. Images were captured at 4× magnification. Presented are (a) the percentages of the total cell count in each of the groups compared to the control group along with their SEM and (b) the representative images of cells from donor RPD5.*

Based on the data we obtained in our experiments, we can conclude that the suitable electric field strengths for successful electroporation, where we can safely say that cells still maintain high enough viability are in the range from 1,0 kV/cm to 1,4 kV/cm. There was also some variability between cells of different donors in electric field strengths needed for electroporation. Using the cell viability assay, we did show that there is a high number of viable cells even in groups that were exposed to higher electric field strengths, though the number of cells and their metabolic activity both seem to drop significantly in the electric field strengths of about 1,8 kV/cm.

The experiments we have conducted thus far offer valuable insight into the potential of applying electroporation inducing electric pulses to primary human chondrocytes and its potential in cell electrofusion. The next step in our research work should be establishing a similar baseline for our other prospective fusion partner – MSCs with the aim of eventual establishment of viable hybrid cells between human MSCs and chondrocytes.

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