URSI GASS 2023, Sapporo, Japan, 19 – 26 August 2023

Use of Microsecond Electric Pulses in Human Mesenchymal Stem Cells for Tissue Regeneration

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Abstract

Nowadays, an optimal treatment for spinal cord injury (SCI) has not yet been reached, so it remains a challenge for researchers. The use of stem cell transplantation has been extensively studied and provides benefits having therapeutic potential. In this way, Mesenchymal Stem Cells (MSCs), have been shown to be able of providing a suitable microenvironment for tissue regeneration and there is an available source of stem cells in each human body. However, the low cell survival and lack of neural differentiation after transplantation require the use of complementary strategies. For that, the main objective in this work is to use microsecond pulse (µsPEF) stimulation, never used previously for this purpose, to enhance the proliferation and neural differentiation of these cells. In addition, calcium signalling is closely related to the biological processes of differentiation and proliferation. Therefore, we have studied the calcium profile of these cells during the neural differentiation process in order to control the calcium oscillations with µsPEF and thus perform some electrical protocols to induce a differentiation or proliferation process that can be used to optimize cells transplantation.

1 Introduction

Spinal cord injuries remain a significant therapeutic challenge due to the inability of the central nervous system to regenerate lost neurons and restore functional connections. The difficulty of the neuronal restoration after SCI is based on the complex cascade of events that inexorably cause a degenerative chronic stage mainly favoured by the non-permissive environment and limited capacity for axonal regrowth. Multifaceted strategies are considered the unique solution for functional restoration by including cell substitution, neuroprotection and axonal growth promotion. Currently, our laboratory research, pursues introducing a new combinatory therapy employing high voltage µsPEF stimulations and low amplitude direct currents on a combination of stem cells transplantation. In this way, MSCs are considered a competent cell source for tissue engineering applications. The state of art about transplantation into SCI has reported an immunoregulatory effect on the tissue that contributes to structuring regenerative microenvironments in injured areas and has the potential to generate multiple differentiated progenies [1]. However, the low survival rate and uncontrolled graft differentiation requires the use of combination therapies. In this context, cell-based therapies still need improvements such as the enhancement of the cell survival and the neuronal differentiation and maturation, by complementary treatments like electrical stimulation. This allows us to alter the niche signals of MSCs to promote proliferation and differentiation towards specific lineages without the use of any exogenous chemical compound.

Furthermore, activation of calcium signalling is essential in neuronal development, including neuronal induction [2] and regulates physiological functions in the cell such as proliferation and differentiation [3]. It was observed that the MSCs naturally exhibit spontaneous calcium oscillations [4] whose frequency varies over the course of differentiation processes. In our case, we are interested in the changes that might be triggered by µsPEF stimulation inducing cellular proliferation or differentiation events. Downstream of these stimuli, calcium oscillation frequency and/or amplitude can give important information, subsequently decoded by some proteins in the cell and whose activity is calcium-sensitive, resulting in "direct" action of effector proteins or activation of gene transcription [5]. Here in the laboratory, it previously has been demonstrated that calcium signalling can be modified by using electrical stimulation [6]. Thus, in our work we try to define some protocols to induce changes in the normal profile of calcium oscillation of MSC to control different processes focused on proliferation and neural differentiation of the cells.

2 Materials and Methods

Human MSC culture: human MSCs (hMSCs) were isolated from human lipoaspirate with consent of the patient (Hospital Gustave Roussy). Cells were grown in MEM Alpha Medium + Glutamax (GIBCO) supplemented with 10% foetal bovine serum (Sigma Aldrich) and 100U/ml penicillin and 100 mg/ml streptomycin (GIBCO) incubated in 37 ºC and 5%CO_2 in a saturated humid atmosphere. To perform the *proliferation experiment* 3 000 cells/cm^2 were seeded and let in the incubator for 2 days allowing them to reach 50% confluency. After that, we performed µsPEF stimulation protocol explained in the section « Analysis of

cell proliferation » taking pictures of the entire Petri dish uncovered by Polydimethylsiloxane (PDMS) mask, every day for 5 days, in brightfield and in fluorescence to detect the nuclei marked with Hoescht 33342 (0.2 µg/ml). To do the *differentiation experiments* 5 000 cells/cm^2 were seeded. After they reached 80% confluency, we induced a 3 step-neural differentiation protocol adapted from Urrutia *et al*. [7]. In neural specification (step 1), cells were grown in α MEM supplemented with: 0,25X B27, 1X N2, 20 ng/mL EGF and 20 ng/mL basic FGF for 5 days. After that, we induced the neuronal commitment (step 2) by changing the media to α MEM supplemented with 0,25X B27, 100 ng/mL Sonic HedgeHog, 2,5 µM, Retinoic Acid and 1 mM AMPc during the next 10 days. Finally, we induced neuronal differentiation (step 3) adding 30 ng/mL BDNF during the final 3 days.

Calcium monitoring and analysis of calcium oscillation: Calcium oscillations were monitored in hMSC during neural differentiation at different time points. Prior to microscopy recording, cells were incubated with Fluo-4 AM (λex = 494 nm / λem = 506 nm) (Invitrogen, Courtaboeuf, France) at 5 µM and Hoechst 33342 (λex = 361 nm / λem = 497 nm) (Sigma Aldrich) at 0,2 µg/mL for 30 min at 37°C, 5% CO_2 in the conditioned medium to avoid inducing any perturbations. Axio Observer microscope (Zeiss, Marly le Roi, France) was used to record calcium movies taking pictures every 10 seconds for 15 minutes in 37ºC and 5% CO_2 conditions. Time lapse microscopy movies were analyzed with CellProfiler image analysis software to provide raw data of Fluo-4 fluorescence intensities linked to cytosolic calcium concentrations. These data were subsequently analyzed with a modified version of the "Spectral Analysis of calcium oscillations" Matlab program made by Uhlén [8] to extract principal frequencies of calcium signals and monitoring the intensity of the calcium against the time.

Electrical pulse generator: To perform the stimulations, cells were previously seeded in an exposure chamber composed of p35 Petri dish (Corning, Boulogne-Billancourt, France) in which a custom-made PDMS mask was used to create a rectangular chamber with desired dimensions based on the setup described in reference [9] showed in Figure 1. The generator used for this experiment was built in University of Zaragoza, Spain with several capacitor banks working as multiplicators able to multiply the voltage of the DC source. This generator can deliver electric pulses whose durations range from µs until ms and voltages reach 900 V. To stimulate cells, grade 2 titanium electrodes were used based in previous results of the laboratory in order to avoid electrochemical reactions and release of metallic particles in the cell medium.

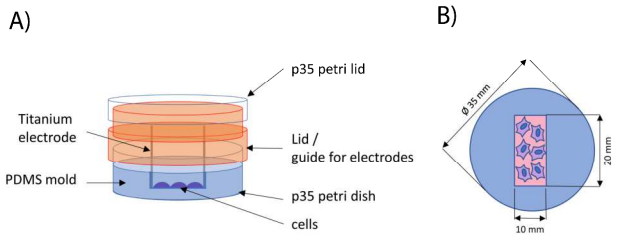

Figure 1. Exposure chamber system p35 Petri dish. A) Perspective view of the entire exposure chamber. B) Top view of cells seeded in the Petri dish with the PDMS mask. Designed by F. André and L. Vallet.

Analysis of cell proliferation: For this experiment, 4 different stimulation conditions were set based on previously obtained results. Using 25 µs bipolar pulses at 300 V/cm, this stimulation was repeated in the following ways: a) 1 pulse every 24 hours, b) 1 pulse every 12 hours, c) 10 pulses at a frequency of 1 pulse per minute every 24 hours and, d) 30 pulses at a frequency of 1 pulse per minute every 24 hours. To quantify the proliferation rate of the cells, brightfield and nuclei-staining images were taken with Cytation™ 1 every 24 hours before stimulation for 5 days. The cells previously, were incubated 30 min with Hoescht 33342 and after that, individual pictures were taken of a 2x1 cm area corresponding to the area where the cells are seeded in the exposure chambers. The pictures were taken at 4X magnification. The nuclei of the cells were quantified using ImageJ software and the number of cells obtained each day was normalised by the number of cells obtained on the first day before starting the stimulations.

3 Results and Discussion

Human MSCs are known to present spontaneous calcium oscillations implying calcium channels and pumps of the plasma membrane and the endoplasmic reticulum [4]. These oscillations regulate many basic functions in the cell such as proliferation and differentiation. Therefore, the possibility to mimic or regulate these oscillations might be useful to regulate MSCs biological functions. In our study, we first started studying the calcium oscillation profile of these cells during the differentiation process. As we can see in Figure 2A, before neural differentiation started (day 0), the cells show a varied frequency profile, with cells oscillating at frequencies above 45 mHz and most of cells oscillating at frequencies between 10 and 18 mHz. During the differentiation process, the profile of oscillating cell populations shifts to the left of the graph, that means cells show calcium oscillations with decreasing frequencies. Before differentiation, the cells oscillate with an average frequency of around 17 mHz. However, at the end of differentiation, the average frequency at which the cells oscillate is around 2.5 mHz, with 19 mHz being the frequency at which they oscillate after the same time of differentiation with a normal proliferation medium (Figure 2B). With these results, we can conclude that to induce neural differentiation we have to decreased the calcium

oscillation frequency of MSC, while to enhance proliferation, we have to increase the frequency.

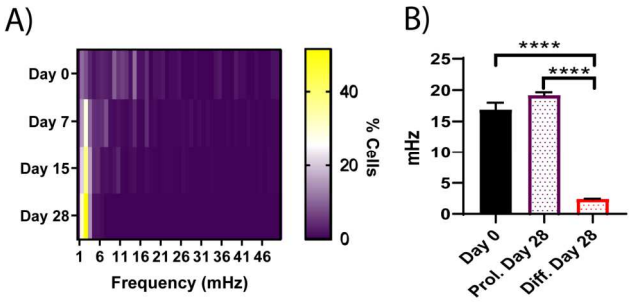

Figure 2. Pattern of calcium oscillations frequency during neural differentiation. A) Heatmap representation of the percentage of the cells oscillating at different mHz. In Some selected points of the experiments are presented in the graphs to show the differences. B) Bar graphs show the mean frequency of calcium oscillation before and after neural differentiation (black and red respectively). As a control for the differentiation, the mean frequency of cells after growth under proliferative condition is shown after 28 days (purple bar). Results were assessed for normality using the Shapiro-Wilk test and one-way ANOVA with Tukey post hoc test, **** $p<0.0001$.

Furthermore, the aim of this study is to control calcium oscillations by using µsPEFs as an effective tool to modulate cytosolic calcium concentrations. Previous results in the laboratory showed that administration of µsPEFs can add or cancel cytosolic calcium spikes, providing a new tool that allowed us to mimic and regulate calcium oscillation in these cells [6]. To do so, we performed a high-throughput experiment by modifying both the pulse duration (between 10 and 100 µs) and electric field (between 100 and 500 V/cm). We defined as an optimal condition first, the result with which we obtained an effect of the pulse on the cell, that means the addition of a peak with the pulse in more than 80% of the cells. From the conditions that passed the first criteria pointed, we searched for the condition that increased calcium oscillations after the pulse in the majority of cells. As a result, we found that the 25+25 µs (bipolar pulse) condition with 300 V/cm fulfilled both criteria because 98% of the cells can respond to the pulse and 48% of them increased their calcium oscillation frequency.

Focusing on increasing cell proliferation by increasing calcium oscillations, we used the 25+25 µs pulse (bipolar pulse) with 300 V/cm as target. For 5 days the cells were subjected to different stimulation conditions using this pulse with different frequencies every 24 hours. Daily pictures were taken during the whole process, where the number of cells was quantified day by day and normalised with the corresponding control at day 0, before starting the stimulations. Being these preliminary results, we can observe one pulse every 12 hours (blue), apparently increasing this growth with respect to the control following the same trend than control group (black). On the other hand, the 30-pulse condition with a frequency of one pulse per minute every 24 hours (red), seems to be the most promising for exponentially increasing cell growth compared to control group growth (Figure 3). However, this experiment needs to be further validated in the laboratory to obtain a final conclusion as well as to corroborate the effect of calcium oscillations during the experiment.

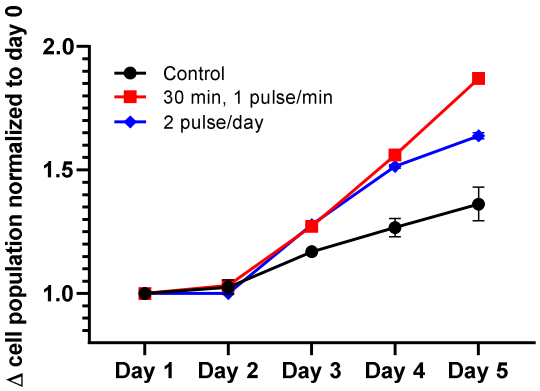

Figure 3. Cell proliferation rate after electrical stimulation. The graph shows the difference in growth from day 0 in each condition over 5 days. Statistical analysis has not been applied as the number of biological replicates shown is 2 for each condition.

4 Conclusions and further work

The main objective of this work is to use electrical stimulation to modify biological processes such as proliferation and differentiation in cells that are dependent on calcium oscillations. Throughout the development of these experiments with hMSCs we can conclude that the frequency of calcium oscillations during cell proliferation increases, while during neural differentiation calcium oscillations decrease until they are blocked at the end of the process. In addition, we are developing several stimulation protocols to enhance both proliferation and neural differentiation in hMSCs. Using a short bipolar pulse (25+25 µs) at 300 V/cm, we have been able to increase the oscillation frequency of hMSCs by almost 50% of the cells. Furthermore, by repeating this pulse every 12 hours, we increased cell proliferation. Results shows that by repeating it 30 times with a frequency of 1 pulse per minute, we were able to increase the proliferation of these cells exponentially and faster in comparison with one pulse every 12 hours.

This work is still in progress, but the targeted changes in the calcium oscillations are now defined and the tools to achieve the desired oscillation patterns are developed. In this context one of the first objectives of our study is to increase the calcium oscillations by using microsecond pulse to increase the proliferation of these cells. This goal is paramount to increase cell survival after transplantation in any tissue. The preliminary data shown are being corroborated by new experiments and complemented by

studies that give us insight into the oscillation profile of the cells during stimulation.

As a secondary objective, due to the experiments presented in this work, we already know that in order to induce neural differentiation of these cells, it is necessary to decrease or even block (given the results with a frequency of less than 3 mHz obtained on the last day of differentiation) the calcium oscillations. Concurrently to the proliferation experiments, we are searching and developing in the same way as we have presented in this work; the pulse that fulfils the conditions that more than 80% of the cells add a spike when the pulse is launched and then decreases or completely blocks the oscillations for at least 5 minutes afterwards [10]. Efforts in this work have been made to implement a new methodology both in the design of the exposure chamber and in the analysis of calcium oscillations. This first approach allowed us to move forward in a faster and easier way that permit us using μsPEFs stimulation to modify intracellular calcium oscillations and thus, modify various biological processes in the cell, thus increasing the potential use of the hMSCs in cell therapy.

Acknowledgements

This research was funded by RISEUP project FetOpen in H2020 Program: RISEUP 964562 H2020 FetOpen program.

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