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

Anti-inflammatory effect of microsecond pulses for spinal cord injury treatment

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Abstract

Spinal cord injury (SCI) is a devastating and common neurologic disorder that has profound influences on modern society from physical, psychosocial, and socioeconomic perspectives.
Currently, despite the numerous studies and clinical trials, the only effective therapy is a clinical approach to reduce the impact of the trauma and physical therapies to keep muscles as much as possible toned.
The onset of a massive inflammatory process constitutes the major impediment to any medical intervention, which has prompted the study of anti-inflammatory strategies applicable.
In this paper, we investigated the anti-inflammatory effect of microsecond electric stimulation, to apply it in SCI treatment.

1 Introduction

The injured spinal cord cannot repair itself, which brings to a devasting condition characterized by a permanent loss of its sensorimotor deficits including paralysis, autonomic dysfunctions, and neuropathic pain.
Spinal cord injury (SCI) is characterized by two distinct phases, which are identified as primary and secondary injury [1]. SCI commonly results from a traumatic impact on the spine that fractures or dislocates vertebrae. The primary injury refers to the first moment of this event, when the initial mechanical forces are applied to the spine. Just after this phase, the secondary injury starts. This includes a subacute and a chronic phase, characterized by an escalating injury cascade, including vascular disruption, inflammation, demyelination and apoptosis, leading to the formation of glial scars and cavities [2]. Above all, the inflammatory response prevents any therapeutic intervention from the outside in this phase and this is why several studies have had the aim of understanding how to block this cascade of events.
Main effectors in the inflammatory response after SCI are macrophages derived from peripheral circulation and from resident microglia. These cells contribute to secondary tissue damage through generation of reactive oxygen species, the production of pro-inflammatory cytokines and other factors [3].
In this paper, we present the results obtained in the framework of RISEUP project (H2020 FET-OPEN grant n.964562) on the effects of microsecond electric pulses stimulation on human microglia (HMC3) and macrophages (U937) cell lines. RISEUP aims at developing an innovative approach for spinal cord regeneration based on microsecond electric pulses (μsec) cells stimulation and one of its tasks is to investigate the possible anti-inflammatory effects of this stimulation in SCI.

2 Methods

2.1 Exposure conditions
Cells (400.000 U937 and 250.000 HMC3; Figure 1 A and B) were plated 24 hours before exposure in 10 cm Petri dishes supplied with a Polydimethylsiloxane (PDMS) pool to enable the inserting of two titanium electrodes placed at 1 cm distance (Figure 2).
Just before pulses delivery, the medium inside the electrodes, only for HMC3 who grow adherent to the plate, was replaced, in order to have a volume of medium of 2 ml maximum.
The pulses generator used for these experiments was supplied by Leroy Biontech (Figure 3).

The pulses stimulation condition was:

- Bipolar pulse: **100 µs + 100 µs**
- Intensity: **250 V/cm**
- 2 pulses / min
- Duration: 30 min

All the biological analysis were performed 24 hours after the exposure.

2.2 Cell proliferation and viability

To estimate the proliferation and to contemporary evaluate the viability, cells were counted through Burker's chamber, after the staining with erythrosine B, a dye who detects dead cells

2.3 Cell cycle analysis

HMC3, that grow as adherent cells, were harvested by trypsinization and collected with floating ones. U937 grow in suspension culture, so they were collected by centrifugation. The pool was washed twice in PBS, then fixed in ice-cold ethanol 80% (1 × 106 cells/ml) overnight. An aliquot of the suspension (at least 5 × 105 cells) was then washed twice in PBS and stained with PI (50 µg/ml) in a mix containing RNAse A (50 µg/ml), Triton X-100 (0.1%), and EDTA (0,1 mM) in PBS, in the dark, for 60 min at room temperature, then immediately analyzed. A FACScan flow cytometer (Becton Dickinson, Bedford, MA, USA), equipped with a 488-nm argon laser, was used for the flow cytometric analyses. The evaluation of cell cycle distribution by DNA content analysis was performed by the FlowJo software.

2.4 Gene expression

Total RNA was extracted with Direct-zol RNA Miniprep (Zymo research) and 500 ng of RNA were converted in cDNA by TaqMan reverse Transcription Reagent (Applied Biosystem) according to manufacturer's indications. 25 nanograms of cDNA were amplified with specific primers using SYBR Green master mix (Thermo Fisher Scientific) and a 7500 Real-Time PCR system (Thermo Fisher Scientific).

3 Results

We started by analyzing the cell response to the stimulation protocol described in methods section. We performed routinely analysis in order to assess if the µsec pulses exposure could affect cell proliferation and viability. By counting cells using erythrosine B, that stains dead cells, we did not observe any difference in number of exposed cells compared to sham, nor for HMC3, nor for U937.

Then, we evaluated if pulses stimulation could affect any step of the cells cycle, but any modulation was detected for both cell lines (Figure 4).

Then we assessed the expression of genes involved in cell proliferation (*c-myc, mki67*) apoptosis (*bax, bcl2*), cell cycle (*ccnd1, ccne1, e2f1*), inflammation (*arg1*, *cox2*, I*l8*). The only gene which was modulated by µsec pulses stimulation was *arg1* in U937 cells.

4 Conclusions

The stimulation protocol described here is the first we used in RISEUP project, in order to investigate the response to µsec pulses of immune system cells, that, as far as we know, have never been subjected to this kind of stimulation before. Currently, the efficacy of µsec pulses exposure in inflammatory conditions is under investigation.

5 Figures

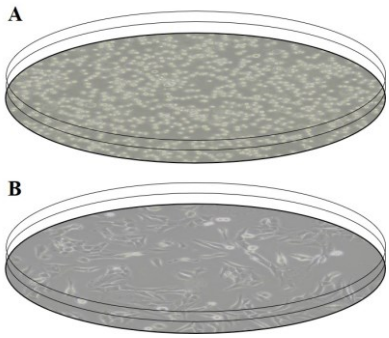

Figure 1. HMC3 (A) and U937 (B) cells. Images were taken by a contrast phase microscope with a 10X objective

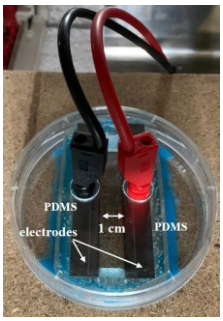

Figure 2. Exposure setup. PDMS "pools" are placed inside a 10 cm petri dish, to enable the insertion of 2 titanium electrodes implanted at 1 cm distance. Cells are plated inside the electrodes

Figure 3. Leroy Biontech **p**ulses generator

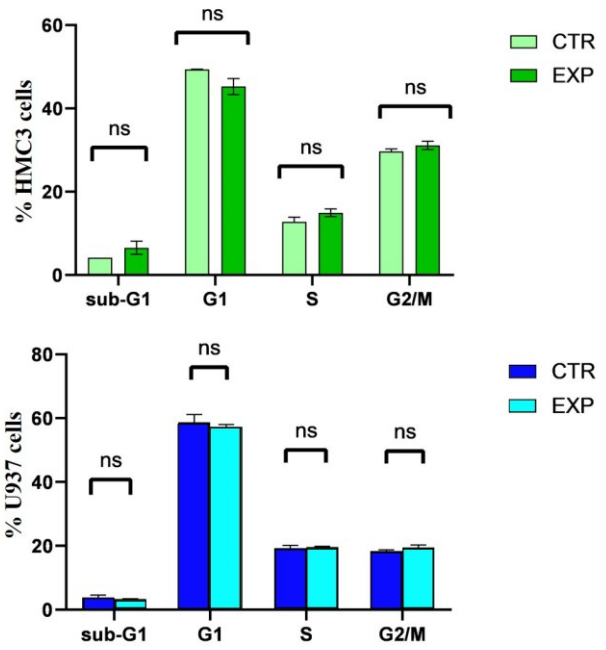

Figure 4. HMC3 and U937 cell cycle analysis

Acknowledgements

This project is supported by FET-OPEN H2020 grant. Grant number 964562

References

[1] S. Li, H. T. P. Dinh, Y. Matsuyama, K. Sato, S. Yamagishi, "Molecular Mechanisms in the Vascular and Nervous Systems following Traumatic Spinal Cord Injury", *Life (Basel)*, **13**, 1, 2022, doi: 10.3390/life13010009.

[2] A. Alizadeh, S. M. Dyck, S. Karimi-Abdolrezaee, "Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms", *Frontiers in Neurology*, 10, 2019, doi: 10.3389/fneur.2019.00282.

[3] S. David, A. Kroner, "Repertoire of microglial and macrophage responses after spinal cord injury", *Nature Review Neuroscience*, **12**, 2011, 388–399, doi: https://doi.org/10.1038/nrn3053