COST EMF - MED (Action BM1309):
European network for innovative uses of EMFs in biomedical appl

Claudia Consales' STSM Report:

STUDY OF THE OXIDATIVE AND EPIGENETIC EFFECTS INDUCED BY SHORT PULSES NEURONAL LIKE CELLS

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Abstract:

Application of high voltage short electric pulses has attracted attention as a unique tool in life sciences, especially in cancer treatment, but the molecular mechanisms of their action on living organisms has not been yet fully elucidated.
During this STSM I explored the biological responses of the SH-SY5Y cells, a neuronal like cell line, exposed to high voltage short electric pulses. In particular, four different types of voltage pulses lasting 100 microseconds and two different types of voltage pulses lasting 10 nanoseconds were applied to these cells.
Cells responses were evaluated after a single pulse by analyzing cells redox metabolism modulation, apoptosis induction and microRNA expression.

A. Purpose of the STSM

The effects of pulsed electric fields on biological cells and tissues have been studied for almost sixty years. The results have brought to different therapeutic applications of them, mainly based on their ability of permeabilizing the cell membrane (Joshi and Schoenback, 2010; Breton & Mir, 2012).
Despite the large number of studies about short high voltage pulses and their use in medical applications, which molecular pathways they activate inside the cells are still not perfectly clear. So, the aim of this STSM was to investigate molecular and cellular responses of the neuronal-like SH-SY5Y cells to single pulses of microsecond and nanosecond duration, comparing results to the recently published biological effects of very long exposures (6h to 72h) to 1 mT 50 Hz magnetic fields (actually to the currents generated in the Petri dish by this sinusoidal magnetic field) (Benassi et al, 2016). To assess a possible comparison, in

terms of effects, between the exposures performed with the magnetic field and the electric pulses, the pulses characteristics were chosen in order to have an energy density identical to the energy density (about 0.015 J/m^3) delivered during the long magnetic field exposures. Electric pulses of higher energies were also used in order to globally evaluate the cell responses.

B. Work Description

SH-SY5Y is a neuroblastoma cell line widely used as neuronal model since these cells display specific neuronal features, such as synaptic structures and functional axonal vesicle transport, and express neuro-specific markers, such as the nuclear protein NeuN, the neuron specific β-III tubulin and the synaptic protein Sv2 (Agholme et al., 2010).
In a previous work, ENEA's group demonstrated that exposing these cells to 50 Hz magnetic field induces a redox homeostasis modulation and increases their sensitivity to the parkinsonian toxin MPP+ (Benassi et al., 2015)
Six different conditions for short pulses (both in the microsecond and nanosecond scale) were analyzed during this STSM; they are described in the table 1 below:

CONDITIONS	APPLIED VOLTAGE Volts (at generator)	ELECTRODE DISTANCE (cm)	ENERGY DENSITY(J/m^3)	INDUCED E FIELD (V/m) (between the electrodes)
1 pulse of 100 µs	0.1	1	0.015	10
1 pulse of 100 µs	10	1	150	1000
1 pulse of 100 µs	600	1	540000	60000
1 pulse of 100 µs	1000	1	$1.5x10^6$	100000
1 pulse of 10 ns	10	1	0.015	1000
1 pulse of 10 ns	1000	1	150	100000

In order to stimulate adherent cells, polydimethylsiloxane (PDMS) holders (named "pools") were fabricated. Using these holders, the cells can be suitably plated in sterile conditions and the electrodes can be placed at the proper distance in a repeatable way. The area delimitated by these "pools" is 5×1 cm^2 allowing a total cell plating of nearly $5×10^5$ cells needed to carry on the proposed endpoints, see Fig.1 and 2 for PDMS pools detail and the experimental arrangements. By this way, we could stimulate cells when they were adherent to the dish, in a condition as much as possible close to the physiological one.

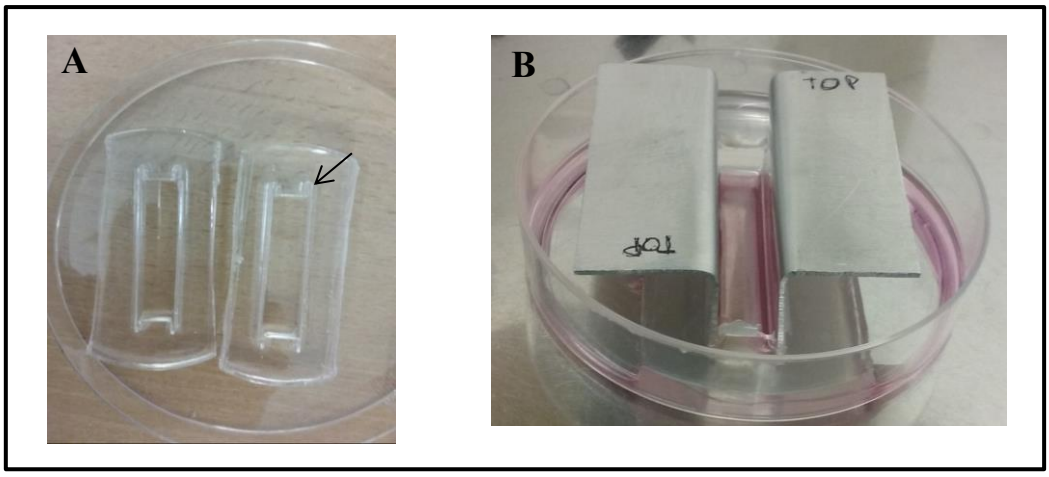

Fig.1: A) PDMS pools. It is possible to observe the fissures (black arrows) inside which the electrodes are placed. B) electrodes placed inside the pools, with the cell culture medium.

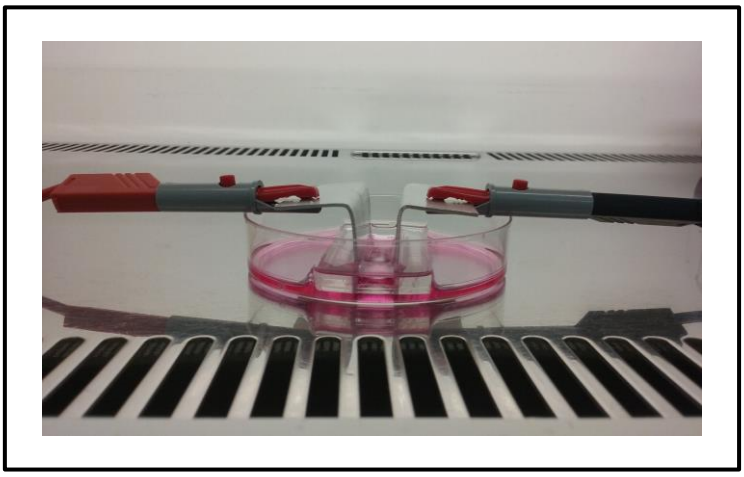

Fig.2: Cells under stimulation within the conceived PDMS arrangement**.**

Cells were plated in the pools two days before stimulation, to let them recover after the culture passage, and the biological analysis were performed at different times after the stimulation, according to which endpoint was evaluated.

In detail:

1) ROS production was assessed, through cytofluorimetric assay, just after stimulation (T0) and 1, 6 and 24 hours after the exposure to the pulse.
2) microRNA expression was analyzed, by Real-time PCR, 1, 6 and 24 hours after the pulse
3) apoptosis was appraised, through western blot analysis, 24 and 48 hours after the pulse.

References

Agholme L, Lindström T, Kågedal K, Marcusson J, Hallbeck M. An in vitro model for neuroscience: differentiation of SH-SY5Y cells into cells with morphological and biochemical characteristics of mature neurons. J Alzheimers Dis. 2010;20(4):1069-82.

Benassi B, Filomeni G, Montagna C, Merla C, Lopresto V, Pinto R, Marino C, Consales C. Extremely Low Frequency Magnetic Field (ELF-MF) Exposure Sensitizes SH-SY5Y Cells to the Pro-Parkinson's Disease Toxin MPP(.). Mol Neurobiol. 2016 Aug;53(6):4247-60.

Breton M, Mir LM. Microsecond and nanosecond electric pulses in cancer treatments. Bioelectromagnetics. 2012 Feb;33(2):106-23.

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C. Results

At molecular level, one of the main results we obtained is that the highest voltage for the microsecond pulse seems to induce an increase in ROS production. With this stimulation morphological changes of cells were also observed (phase contrast images were acquired 24 hrs after the pulse delivery using a Zeiss Observer Z1 and a 40× objective). In particular, cell swelling and fusion were observed (Fig.3).

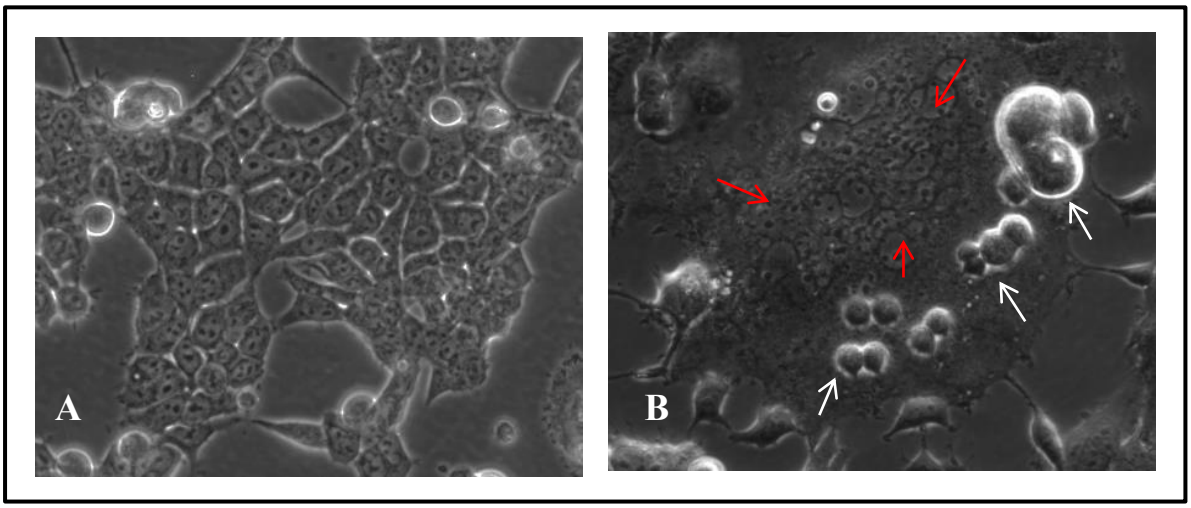

Fig. 3: SH-SY5Y 24 hrs after (B) or not (A) a stimulation with one pulse of 100 µs at 1000 V.
Control cells (A) appear well separated with an intact membrane and cell morphology is remarkably well preserved. In pulsed cells, instead, cells boundaries are lost and it is not possible to distinguish one cell from the other (red arrows). Furthermore, big bulges originated from cells are clearly visible (white arrows)

To further verify cell permeabilization after the application of pulses at high voltages (i.e. 1000 and 600 V) YO-PRO-1 dye was used and its emission observed by fluorescence microscopy (Yo-Pro-1 $\lambda_{emission}$=510 nm).
Cells were tripsinized and then re-suspended in DMEM, then Yo-Pro-1 was added 5 minute before the acquisition of the first image at a final concentration of 3 µM.

Images were acquired with an inverted microscope (Zeiss Observer Z1), with an exposure time of 300 ms and a 20× objective). Images were acquired 2 minutes before the pulses delivery and 2 and 5 minutes after the exposure (Fig. 4). Three independent experiments were performed. Control cells were also imaged.

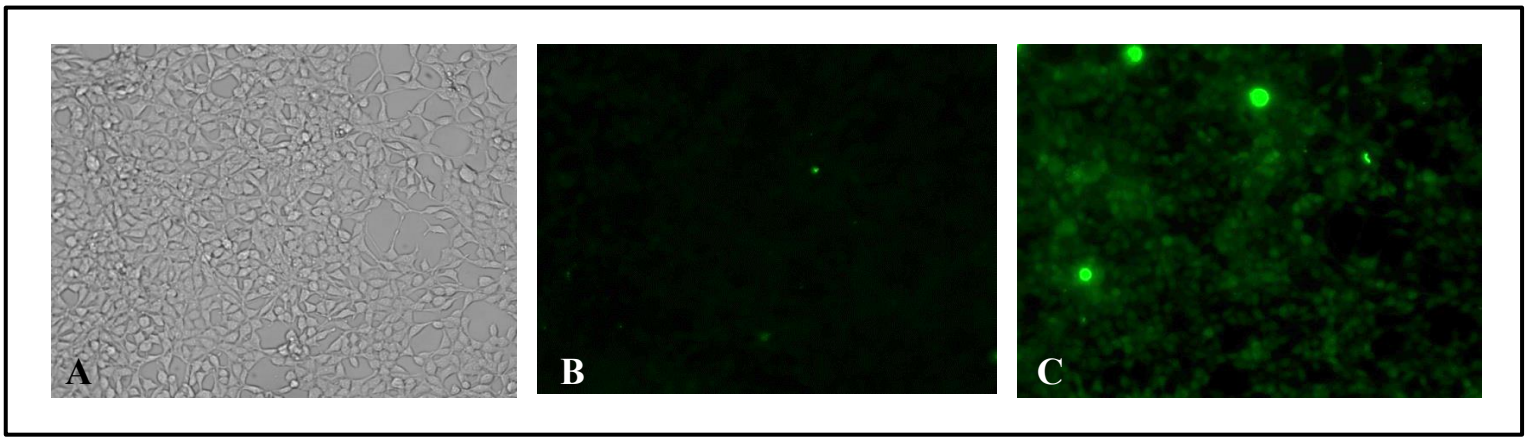

Fig. 4: SH-SY5Y before and after a 100 µs pulse at 1000 V.
A) cells observed with bright field. B) cells stained with Yo-Pro-1 before pulse stimulation. C) cells stained with Yp-Pro-1 5 minutes after pulse stimulation. A clear cytoplasmic Yo-Pro-1 signal is visible, indicating that the pulse induced cell permeabilization

As shown in Figure 4, 100 µs pulse at 1000 V could induce cell permeabilization and the same result was obtained by applying a 100 µs pulse at 600 V (data not shown). All the lower intensity conditions weren't able to induce Yo-Pro-1 internalization into the cells (data not shown).
Results analysis from epigenetic and apoptosis experiments are still ongoing, in order to check for every statistically significant change.

D. Future collaboration with host institution

The collaboration among ENEA and Gustave Roussy, i.e. among Dr. Carmela Marino and Dr. Lluis M. Mir, has started a lot of years ago, and has been strengthened after Dr. Claudia Consales' visit at Dr. Mir's Laboratory.
A joint participation to different EU and French-Italy calls is expected.

E. Expected Publications

During this STSM the molecular response of different pulses, in terms of energy and duration, has been evaluated, and, after the statistical analysis, all the results will be published in a peer review journal

Confirmation by the host institution of the successful execution of the STSM:
We confirm that Dr. Claudia Consales has performed the research work as described above.

Contact Person of Host Institution	Name of researcher
Dr. Lluis M. Mir	Claudia Consales
Signature	Signature