# CALCIUM ELECTROCHEMOTHERAPY USING ULTRA-FAST NANOSECOND ELECTRIC PULSES AND THEIR EFFECTS ON MITOCHONDRIA TRANSMEMBRANE POTENTIAL AND OXIDATION

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Electroporation refers to the phenomenon wherein the application of high, short-duration pulsed electric fields (PEF) to biological cells induces a transient increase in plasma membrane permeability [1]. The pulse duration of nanosecond PEF (nsPEF) is significantly shorter than the time constant of biological cell membrane and can penetrate into the interior of the cell and organelles inducing various biological effects [2]. Thus, nsPEF alter mitochondrial function by permeabilizing its membrane. Mitochondria must maintain the electric potential across the inner membrane and a loss of the proton gradient often leads to the disruption of the mitochondrial membrane potential (MMP) and couples with the ROS-induced oxidative stress [3]. Both of these signalling systems profoundly influences the rate of ATP synthesis [4], which is also linked to calcium overload [5]. Mitochondrial calcium overload also work complementarily with ROS overproduction and dependent on the mitochondrial membrane potential [6]. All of these processes ultimately causes cell death involving apoptosis or necrosis. Therefore, stimulation of apoptosis through a mitochondrial-mediated pathway with nsPEF could enhance current electrochemotherapy protocols.

Pulses were generated using a custom-built high-frequency (up to 6.6 MHz) square-wave generator (≤3 kV). The voltage varied in 50 V – 1.6 kV range, corresponding to a 0.5–16 kV/cm electric field in the cuvette. We applied 50 ns or 300 ns monophasic pulses (100 pulses/burst, 100 ns delay) and a microsecond reference protocol (1.2 kV/cm × 100 µs × 8, 1 Hz). CHO-K1-Luc cells were used as a model. Permeabilization was detected with Yo-Pro-1, MMP disruption with TMRM, ROS with MitoSOX Red, ATP depletion via D-luciferin oxidation, and viability via PrestoBlue. For CaECT, $CaCl_2$ (5 mM) was added in low-conductivity HEPES buffer (10 mM HEPES, sucrose, 1 mM $Mg^{2+}$).

High permeabilization (>75 %) was achieved with pulsed electric field exceeding 6 kV/cm for 50 ns pulses and 4 kV/cm for 300 ns (n = 100), respectively. PEF amplitude was limited to 6–12 kV/cm protocols to ensure overlapping points in terms of permeabilization efficacy for both 50 ns and 300 ns protocols. 300 ns pulses caused a greater MMP reduction than 50 ns pulses, indicating that high permeabilization is required to trigger significant depolarization of mitochondria within the studied parametric range. In the context of ROS, 300 ns pulses trigger detectable oxidation of mitochondria even without added calcium. When calcium is added in both cases (50 ns and 300 ns) the oxidation of mitochondria increases up to 2000 %. A viability assay showed that 300 ns pulses at >6 kV/cm caused partly irreversible electroporation and cell death in all CaECT protocols, while 50 ns pulses (6–12 kV/cm) induced reversible electroporation. For 50 ns pulses, 10–12 kV/cm enabled sufficient calcium electrotransfer for effective CaECT in vitro.

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