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Different Cell Viability Assays Reveal Inconsistent Results After Bleomycin Electrotransfer In Vitro

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Abstract The aim of this study was to compare different and commonly used cell viability assays after CHO cells treatment with anticancer drug bleomycin (20 nM), high voltage (HV) electric pulses (4 pulses, 1200 V/cm, 100 μs, 1 Hz), and combination of bleomycin and HV electric pulses. Cell viability was measured using clonogenic assay, propidium iodide (PI) assay, MTT assay, and employing flow cytometry modality to precisely count cells in definite volume of the sample (flow cytometry assay). Results showed that although clonogenic cell viability drastically decreased correspondingly to 57 and 3 % after cell treatment either with HV pulses or combination of bleomycin and HV pulses (bleomycin electrotransfer), PI assay performed ~15 min after the treatments indicated nearly 100 % cell viability. MTT assay performed at 6–72 h time points after these treatments revealed that MTT cell viability is highly dependent on evaluation time point and decreased with later evaluation time points. Nevertheless, in comparison to clonogenic cell viability, MTT cell viability after bleomycin electrotransfer at all testing time points was significantly higher. Flow cytometry assay if used at later times, 2–3 days after the treatment, allowed reliable evaluation of cell viability. In overall, our results showed that in order to estimate cell viability after cell treatment with combination of the bleomycin and electroporation the most reliable method is clonogenic assay. Improper use of PI and MTT assays can lead to misinterpretation of the experimental results.

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Introduction

Electroporation is a well-established method that employs the use of high voltage (HV) electric pulses to facilitate intracellular delivery of various molecules and ions (Weaver and Chizmadzhev 1996; Rols and Teissié 1990; Yarmush et al. 2014). Electroporation takes place when the cells are exposed to external electric field, strong enough to induce over-threshold transmembrane potential (Weaver and Chizmadzhev 1996; Hibino et al. 1993; Valic et al. 2003). Electroporation efficiency is highly dependent on electric field strength pulse duration, number of pulses, cell size and shape as well as cell orientation in electric field (Hibino et al. 1993; Pucihar et al. 2008; Zou et al. 2015). It is believed that electroporation-induced increase in membrane permeability is compatible with formation of aqueous pores in the lipid phase of the membrane (Weaver and Chizmadzhev 1996; Böckmann et al. 2008; Venslauskas et al. 2009). The molecular dynamics simulations seem likely to support this general theory (Casciola et al. 2014; Bennett and Tieleman 2014), nevertheless the definitive proofs are still needed (Teissie 2014).

Soon after acknowledging of electroporation as a tool for targeted molecule delivery, it was exploited for intracellular transfer of anticancer drugs and DNA (Kinosita and Tsong 1978; Neumann et al. 1982; Mir et al. 1988; Labanauskiene et al. 2009; Vásquez et al. 2012). The feasibility of electroporation to enhance delivery of anticancer drugs into cells and tissues made possible the development of antitumor electrochemotherapy (ECT)

(Mir et al. 1991), lately optimized in in vivo studies (Serša et al. 1995; Miklavčič et al. 1998; Šatkauskas et al. 2005; Soden et al. 2006; Corovic et al. 2008; Labanauskienė et al. 2009) and implementation in clinics (Belehradek et al. 1993; Mir et al. 2006; Edhemovic et al. 2014).

Aiming to delineate mechanistic details of electroporation and also to implement various amendments to achieve more efficient or controllable cell electroporation and drug delivery, the initial experiments are performed in vitro (Mir et al. 1988; Gehl et al. 1998; Saczko et al. 2014). For example, in recent publications to test potency of calcium electroporation for anticancer therapy, preclinical studies had been first performed and optimized both in vitro and in vivo (Frandsen et al. 2012; Frandsen et al. 2014). In these and similar studies, precise estimation of cell viability is essential to validate any amendment in the experimental protocol.

Nowadays, a high variety of cell viability assays are available that differ on their principles and methodologies (Li et al. 2014; Gumulec et al. 2014). In some cases, misunderstanding of principles of the assays and improper application can lead to misinterpretation of the cell viability results. For example, since there is no uniform perception of the tetrazolium salts metabolism inside the cell in response to various external stimuli on a cell, the discrepancies can appear in determining cells viability using the enzymatic activity-based assays like MTT, XTT, and WST-1. (Berridge et al. 1996). Loose interpretation of such assays together with improper time selection to perform measurements can lead to inconsistency of the cell viability results. This inconsistency can be even more evident taking into account that cell death after bleomycin electrotransfer can result due to immediate cell lysis following the disruption of the membrane by electric pulses or cell death due to bleomycin activity (Mir et al. 1996; Hecht 2000). The inconsistency can also arise because of the differences of cell death dynamics after such treatments.

Therefore, in this study, we aimed to compare different and commonly used viability assays after cell treatment with bleomycin, electric pulses, or combination of bleomycin and HV electric pulses.

Materials and Methods

Cell Culture

CHO (Chinese Hamster Ovary) cell culture line was cultivated in growth DMEM (Sigma-Aldrich, St. Louis, USA) medium containing 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1 % L-glutamine solution (Invitrogen Inc., Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were grown in 96 mm culture dish (TPP, Trasadingen, Switzerland) at 37 °C in humidified 5 % CO_2 atmosphere in the incubator. After trypsinization, cells were suspended in low conductivity electroporation medium [0.25 M glucose, 1 mM $MgCl_2$, 10 mM Na_2HPO_4 (pH 7.4)] at a concentration of 2×10^6 cells/ml.

Exposure of Cells to Electric Pulses and Bleomycin Electrotransfer

Four experimental conditions were employed in the study: (i) control (no treatment), (ii) cells treated with bleomycin (BLM, Nipon Kayaku, Japan) for 1 h at a final 20 nM concentration, (iii) cells treated with 4 HV square-wave electric pulses of 1200 V/cm pulse strength, 100 μs pulse duration, 1 Hz repetition frequency (4 HV group), and (iv) cells treated with BLM and 4 HV pulses (BLM + 4 HV group). Electric pulses were delivered by an electroporator developed in our laboratory in collaboration with Kaunas University of Technology (Čepurnienė et al. 2010). For each experimental point 9×10^4 cells in 45 μL of EP medium were used respectively together with either 5 μL of 200 nM BLM or 5 μL of EP medium. The resulted 50 μl of cell suspension was placed between the plates of stainless steel plate electrodes separated by 2 mm. After sham or the electric pulse treatment in the absence or presence of BLM, cells (50 μl of cell suspension) from the electrodes were removed by gentle tap of the electrodes at the bottom of 40 mm petri dish (TPP, Trasadingen, Switzerland). Since cell viability after each experimental condition was evaluated using four different viability assays, including different time points after treatment (in overall 21 viability measurement), a large amount of cells was needed. This large amount of cells was obtained repeating identical electroporation procedure three times and collecting the treated cells into a single petri dish. Cells then were incubated in petri dishes (if not stated otherwise) for 15 min at room temperature (21 °C) and afterwards supplemented with 850 μL growth medium up to 1 ml.

Evaluation of Cell Viability

Clonogenic Assay (CA)

Following specific cell treatment and incubation in the growth medium, 1×10^4 of cells were taken from 1 ml of the cell suspension, diluted in DMEM, and 400 of the cells were plated into 4.1 cm^2 tissue culture dishes containing 2 ml of growth medium. The cells were allowed to grow for 7 days, then fixed in 1 ml of 96 % ethanol for 10 min, and stained using crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA). The number of cell colonies was

assessed under light microscope MBS-9 (LOMO, St. Petersburg, Russia) and then normalized to the control.

Propidium Iodide (PI) Assay

PI assay was used as a tool to determine cells viability assuming that cells uptake PI due to loss of the barrier function of the membrane. Following specific cell treatment and 15 min incubation and supplementation with 850 µl of growth medium, 2×10^4 of the cells were taken and transferred directly into 1.5 ml tubes. The cell suspension was supplemented with PI at 5 µM final concentration. After 2 min incubation, the percentage of PI-positive (dead) cells was evaluated using BD Accuri™ C6 flow cytometer (Accuri Cytometers Inc. MI, USA). Cell viability was estimated by evaluating the percentage of viable (PI-free) cells and normalizing to the control.

MTT Assay

MTT is used as a colorimetric cell viability assay assuming that cell enzymatic activity can be related to cell viability. Since the metabolic activity can change during the time course after cell treatment, cell viability was evaluated at various time points after the experiment. Consequently, for evaluation of cell viability after 6, 12, 24, 36, 48, and 72 h after the experiment, 2×10^4, 1×10^4, 1×10^4, 6×10^3, 4×10^3, and 1.5×10^3 cells were plated in the wells of 96-well microplates (Plastibrand; Wertheim, Germany). At the specific time point, after the experiment, 20 µl of growing medium was removed from the wells and 20 µl of MTT salt at concentration of 5 mg/ml was added and incubated for 2 h. Afterwards the medium was taken out from the wells, the wells were washed twice with 100 µl of PBS. Formazan formed in the cells was dissolved using 100 µL (≥99.7 %) of isopropanol (Chempur, Poland). All of the resulted content of each well was transferred into the corresponding well of another 96 well transparent microplate and absorbance was measured with Genios Pro Basic W/O FP spectrometer (Tekan, Austria GmbH). Optical density was estimated at 535 nm and these values were corrected by subtracting the optical density at 612 nm as a background. Changes in optical density, corresponding to changes in cell viability, were normalized to the control.

Flow Cytometry Assay (FCA)

Flow cytometry employing BD Accuri™ C6 cytometer provides with a possibility to determine the number of cells with no 'marker' molecules used. This flow cytometer modality was employed in the study to estimate exact number of cells in a specific sample at 2 min, 6, 12, 24, 36, 48, and 72 h time points after the experiment. The 2 min measurements, different from other time point measurements were performed without 15 min incubation after cell treatment. After the experiment, 1×10^4 of cells were plated in 24-well microplate (Plastibrand; Wertheim, Germany). At the defined time point (except 2 min), the cells were trypsinized and resuspended in 200 µl of PBS. Using flow cytometer, absolute number of cells was counted in 50 µl of each cell suspension sample and normalized to the control.

Statistical Analysis

Each experimental condition for each cell viability assay was independently repeated at least four times. All cell viability assays after specific cell treatment were performed from the treatment corresponding pool of the cells collected in a petri dish. The results are represented as mean ± standard error of the mean. The statistical significance of differences between the groups was evaluated by two-sided, unpaired Student's t test (*NS* not significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.005$).

Results

It is well known that BLM transfer into cells using electric pulses results in cell death, which depends on the number of permeabilized cells and BLM concentration (Poddevin et al. 1991). Different research groups evaluate cell death using either cell clonogenic assay (CA) or PI (Kumar et al. 2015) assay. Some others use MTT assay (Gehl et al. 1998; Kaminska et al. 2012). Nevertheless, whether these tests reveal consistent and comparable cell viability results after BLM electrotransfer is unknown. To answer this question, we first set to evaluate cell viability after cell treatment with BLM, 4 HV pulses, and combination of BLM and 4 HV pulses by using CA. This resulted in 100 ± 8.2, 57 ± 15.5, and 3 ± 0.2 % viable cells, respectively, (Fig. 1). In parallel, cell viability was evaluated using PI assay. In contrary to CA, cell viability using PI test remained around 100 % level for all treatment conditions tested (Fig. 1).

In parallel, cell viability was evaluated using MTT assay. Since MTT assay is based on metabolic activity of the cell that can change during the course of the time we performed MTT assay at different time (6–72 h) points (Fig. 2).

As it is seen from the Fig. 2, BLM alone had no significant impact on cells and the cell viability remained close to 100 % for all time points tested. Cell treatment with 4 HV pulses resulted in slight decrease in cell viability that was more evident at longer time points. Indeed, in comparison to cell viability of 95.2 ± 8 % at 6 h, it was 78 ± 8.5 and 69 ± 11.6 % at 48 and 72 h after the treatment,

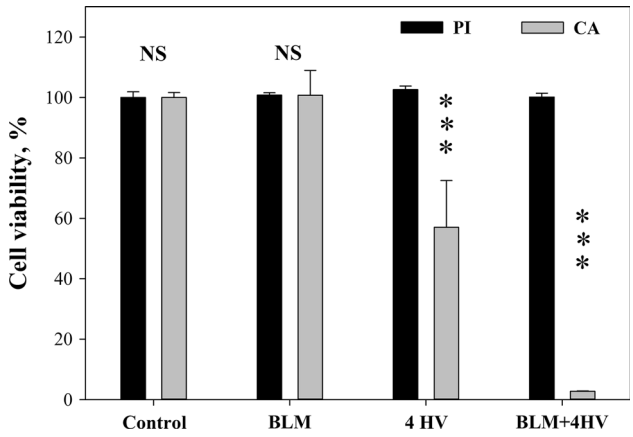

Fig. 1 Cell viability after cell treatment with bleomycin (BLM, 20 nM), four HV pulses (4 HV, 4 × 1200 V/cm, 100 µs, 1 Hz), and combination of BLM and 4 HV pulses assessed by colony formation assay (CA) and propidium iodide (PI) test. Here *NS* not significant, *** $p < 0.001$

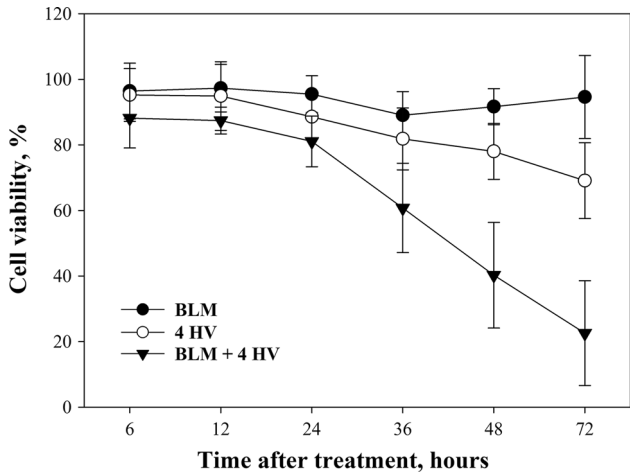

Fig. 2 Cell viability after cell treatment with BLM (20 nM), 4 HV pulses (4 × 1200 V/cm, 100 µs, 1 Hz), and combination of BLM and 4 HV pulses assessed by MTT at various time points after the treatment

respectively. More significant changes in cell viability during the time course were observed after cell treatment with combination of BLM and HV pulses. Although cell viability at 6 h after the treatment was 88 ± 9.1 %, at 72 h it dropped to 22 ± 16 %. These results clearly show that cell viability assessed using MTT test after identical cell treatment can differ significantly, depending on the time of the assessment. In turn, this shows that cell death after treatment with HV pulses or combination of BLM and HV pulses occurs not immediately but during the course of at least several days. To test this assumption, we employed flow cytometry assay (FCA) to calculate the exact number of cells in a definite cell suspension volume at various time points after the treatment (Fig. 3).

Similarly to MTT, FCA revealed that BLM alone did not reduce cell viability. At all testing time points, the number of cells detected by flow cytometry was slightly variable, however was not different from the control. On the other hand, different from MTT, FCA revealed significant cell loss after cell treatment with HV pulses and combination of BLM and HV pulses. Notably, the difference was observed testing cell viability already at 6 h after the experiment. Indeed, at this testing time point, the cell viability dropped to 78.4 ± 5.3 and 71.1 ± 1.9 after cell treatment with 4 HV pulses and BLM + 4 HV, respectively, (Fig. 3). At the later time points, the cell viability after both these treatments tended to decrease and was 48.4 ± 10.0 and 4.8 ± 0.3 at the 72 h testing time, respectively. Thus, in comparison to MTT test, FCA revealed highly reduced cell viability at all testing time points.

Noteworthy, FCA cell viability measured at 2 min remained at 100 % level both after cell treatment with 4 HV pulses and BLM + 4 HV. This indicates that the HV pulses did not induce lethal membrane disruption leading to immediate cell lysis, but rather triggered some longer lasting processes that at 6 h after the treatment led to cell death.

Since CA is directly related to cell viability and consequently provides the most reliable results, the cell viability results obtained using MTT and FCA were compared with that obtained with CA. For comparison, we have selected those testing time points that are most often chosen when MTT test is used (Fig. 4).

Following the cell treatment with BLM alone, both MTT and FCA assays showed comparable results with CA. The cell viability was close to 100 % at all testing time

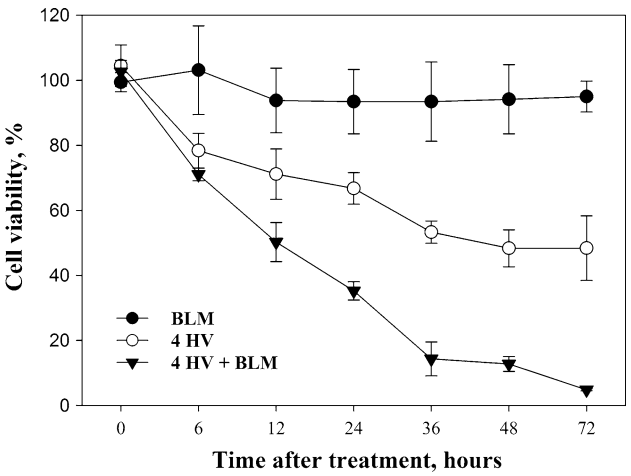

Fig. 3 Cell viability after cell treatment with BLM (20 nM), 4 HV pulses (4 × 1200 V/cm, 100 µs, 1 Hz), and combination of BLM and 4 HV pulses assessed by flow cytometry assay (FCA) at various time points (2 min, 6, 12, 24, 36, 48, and 72 h) after the treatment

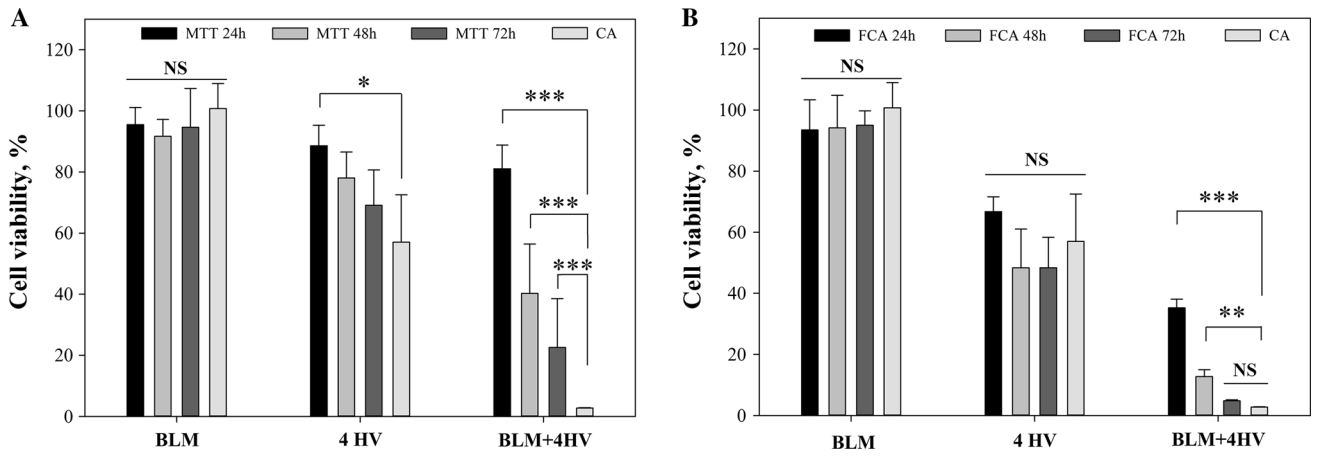

Fig. 4 Comparison of cell viability results obtained using MTT (**a**) and FCA (**b**) at 24, 48, and 72 h with cell viability results obtained using CA. Cells were treated either with a BLM (20 nM), 4 HV pulses (4 × 1200 V/cm, 100 µs, 1 Hz), or combination of BLM and 4 HV pulses. Here *NS* not significant, * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$

points (Fig. 4, BLM bars). Higher variations were obtained comparing these viability assays after cell treatment with 4 HV pulses (Fig. 4, HV bars). As mentioned previously, MTT assay revealed that the obtained cell viability depends on the testing time point. As it is seen in the Fig. 4A, at 24 h, MTT viability results were significantly different from that obtained using CA ($p < 0.05$). The MTT viability at 48 and 72 h started to be comparable with CA ($p > 0.05$). In contrary, cell viability obtained with FCA, despite some variations, at all tested time points was comparable with CA ($p > 0.05$).

Striking differences in cell viability were obtained comparing CA with either MTT or FCA after bleomycin electrotransfer at all testing time points (Fig. 4, BLM + 4 HV bars). MTT assay revealed significantly different cell viability results in comparison to CA with a significance value of $p < 0.01$ (at least) for all testing time points. Notably, at the most often used testing time points, namely 24 and 48 h, the MTT cell viability was correspondingly 79 and 37 % higher in comparison to CA.

Comparing cell viability after bleomycin electrotransfer obtained with CA and FCA, significant difference was obtained only for 24 h time testing point ($p < 0.01$). At this time point, in comparison to CA, cell viability using FCA was 32 % higher. No significant differences between these tests were obtained when FCA cell viability was measured at 48 and 72 h.

Discussion

The choice of cell viability assay after cell treatment with various external stimuli usually depends on the familiarity with the assay, availability of materials or equipment, and convenience of the assay in terms of complexity and assay duration. Despite methodological differences, these assays are considered to provide comparable cell viability results. However, when stimuli of different origin are delivered to cells, the mechanisms of cell death are different and therefore can lead to cell death at various time periods. In case of electrochemotherapy (Mir et al. 1991; Miklavčič et al. 2014), cell death can occur due to irreversible cell electroporation and due to the activity of anticancer drug inside the cell. Depending on the intracellular drug target, the cell can be killed sooner or later. Moreover, irreversible electroporation itself can have several cell killing mechanisms related either with direct and almost immediate membrane disintegration or loss of essential metabolites and/or initiation secondary processes that lead to cell death at delayed times. In the present study, we aimed to evaluate cell viability after cell treatment with anticancer drug bleomycin, electric pulses either in the absence or in the presence of bleomycin by exploiting and comparing four different viability assays.

The results obtained in the present study demonstrated that reliable evaluation of cell viability can be obtained only if the evaluation test is performed at appropriate time point after cell treatment. For example, unexpectedly, PI test showed to be unsuitable for cell viability evaluation after these treatments (Fig. 1). Considering that PI assay is performed within 15–30 min after the treatment the most plausible explanation of the PI assay inaccuracy is that cell death occurs at later times in comparison to PI assay application (Park and Kang 2013). On the other hand, inaccuracy of PI assay can also be explained by the fact that some cells die and disintegrate immediately after the treatment and, therefore, become undetectable using flow cytometry when calculating PI-positive cells. Nevertheless,

this assumption is not supported by FCA performed at 2 min after the treatment.

Importance of choosing proper time for cell viability evaluation is evidenced by both MTT assay and FCA. Indeed, both tests reveal that evaluated cell viability is changing during the course after the treatment (Figs. 2, 3, 4). Considering these results, they demonstrate that different and even contradictory results can be obtained if the viability evaluation time is chosen loosely. For example, FCA clearly shows that cells after HV or BLM + HV pulses are lost continuously starting from 6 h (or even earlier times) up to 48 h for HV pulse-treated cells and up to 72 h after cell's treatment with a combination of BLM and HV (Fig. 3). The cell viability evaluated using MTT at 24 and 72 h was 81 and 22.5 %, respectively, which is much higher than cell viability obtained by CA. At earlier MTT testing times, these results are completely unreliably showing very high cell viability (Fig. 2). The inconsistency of MTT assay at earlier testing time points is substantiated by FCA which displayed significantly lower viability results than MTT both after cell treatment with HV pulses and combination of BLM and HV pulses. Thus, MTT can be used only after 1 or 2 days and even though the test shows the tendency of cell viability but not quantitative values. Similar conclusion is reported by Angius and Floris (2015) who showed that cytotoxicity of drugs vehicled by liposomes evaluated by MTT result in higher cell viability compared with the cell viability estimated by direct counting cells under the microscope (Angius and Floris 2015).

Since the MTT assay is based on the ability of a cell to convert MTT to formazan, it reflects metabolic cell activity rather than direct cell viability. In support to this notion, it was shown that inhibition of DNA expression not necessarily decreases MTT conversion (Berridge and Tan 1993), indicating metabolic activity revealed by MTT assay to be not directly related to cell viability. Since bleomycin inside the cells induces DNA strand breaks ultimately leading to cell death (Miyaki et al. 1973; Mir et al. 1996), metabolic activity can remain high. Indeed, membrane electroporation, together with induction of oxidative stress (Gabriel and Teissie 1995) initiates various processes to restore membrane and cell integrity (Smith and Weaver 2011; Romeo et al. 2013). These processes in turn can activate cell metabolic activity, reflected by MTT, even if those cells ultimately die.

In conclusion, our results show that PI test is not suitable for cell viability evaluation after BLM and electric pulse treatment. MTT shows the only tendency of cell viability, however does not directly reflect the exact number of viable cell and therefore must be used with caution or at later time points after the treatment. FCA, exploiting possibility of flow cytometers of new generation to calculate cell number in precise sample volume provides with the opportunity of evaluation of cell number dynamics in the course of time after the treatment. If used at later times, 2–3 days after the treatment, FCA allows reliable evaluation of cell viability. In overall, our results show that in order to estimate cell viability after cell treatment with combination of cytotoxic agents and electroporation, the most reliable method is cell colony formation assay, despite its long duration.

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