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Enhanced Killing Effect of Nanosecond Pulse Electric Fields on PANC1 and Jurkat Cell Lines in the Presence of Tween 80

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Abstract We investigated the effects of nanosecond pulse electric fields (nsPEFs) on Jurkat and PANC1 cells, which are human carcinoma cell lines, in the presence of Tween 80 (T80) at a concentration of 0.18 % and demonstarted an enhanced killing effect. We used two biological assays to determine cell viability after exposing cells to nsPEFs in the presence of T80 and observed a significant increase in the killing effect of nsPEFs. We did not see a toxic effect of T80 when cells were exposed to surfactant alone. However, we saw a synergistic effect when cells exposed to T80 were combined with the nsPEFs. Increasing the time of exposure for up to 8 h in T80 led to a significant decrease in cell viability when nsPEFs were applied to cells compared to control cells. We also observed cell type–specific swelling in the presence of T80. We suggest that T80 acts as an adjuvant in facilitating the effects of nsPEFs on the cell membrane; however, the limitations of the viability assays were addressed. We conclude that T80 may increase the fragility of the cell membrane, which makes it more susceptible to nsPEF-mediated killing.

Keywords Nanosecond pulse electric field · Tween 80 · PANC1 cell · Jurkat cell

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Introduction

The use of nanosecond pulse electric fields (nsPEFs) for the treatment of tumors is a relatively new and exciting field which is being explored by a number of groups around the world. The charging time constant for a typical mammalian cell (of 10 μm diameter) is in the order of 100 ns (Schwan et al. 1985). PEF exposures with durations of <100 ns can effectively penetrate into a mammalian cell. These ultrashort electric pulses are known as nsPEFs and have been shown to induce apoptosis in HCT116 human colon carcinoma cells, Jurkat cells, B10-2 fibrosarcoma tumors (Beebe et al. 2003a, b) and calcium bursts in Jurkat cells (Vernier et al. 2003). It has been observed that when high-voltage electric pulses of short time intervals (12 kV/cm, 60 ns) were applied to Jurkat, GH3 and PC-12 cells, there was a significant decrease in the membrane potential and increased cell death (Pakhomov et al. 2007). The major advantage of nsPEFs is their unique ability to noninvasively induce apoptosis without generating any huge thermal effect (Nuccitelli et al. 2006). Although nsPEFs significantly decrease tumor volume by 80 % in mice, histological results have shown significant damage to the healthy peripheral skin due to application of high electric fields (Chen et al. 2009). Increases in heart and respiratory rates were also observed when nsPEFs were applied to treat melanomas in mice (Chen et al. 2009). Keeping these characteristics in mind, nsPEFs provide a new approach to physically target tumor cells.

Polysorbate 80 is a nonionic surfactant which is commercially known as Tween 80 (T80) (Zheng and Obbard 2002) and has been used as an adjuvant along with different cancer-treating drugs (Tsujino et al. 1999). The killing effect shown by actinomycin-D and daunomycin on drug-resistant Chinese hamster cells was directly

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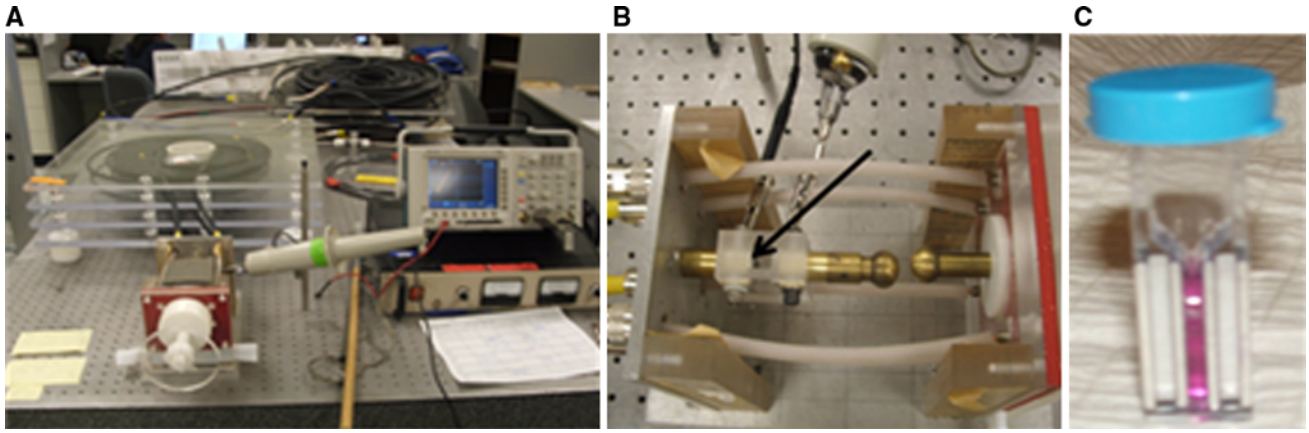

Fig. 1 Experimental setup for nsPEF treatment. **a** Sixty-nanosecond pulse generator. **b** The delivery chamber where electroporation cuvettes are inserted (*arrow*). **c** The 0.2-cm-wide cuvette with cell culture media

proportional to the concentration of adjuvant (T80) used (Riehm and Biedler 1972). T80 also enhances the chemotherapeutic efficiency of drugs such as doxorubicin (Adriamycin) (Chitnis et al. 1984; Parris et al. 1987), VP16 (Tsujino et al. 1999), ethoglucid (Epodyl), mitomycin-C and thiotepa (Parris et al. 1987) by altering the permeability of the plasma membranes of cancer cells in vitro (Riehm and Biedler 1972). These studies show the significance of T80 as an adjuvant in effectively killing cancer cells.

Our present work focuses on enhancing the killing efficiency of nsPEFs by (using T80 as an adjuvant) exposing PANC1 and Jurkat cells to T80. We believe that exposure of cells to T80 compromises the integrity of the plasma membrane of cells, thereby increasing the effect of nsPEFs on intracellular structures of cells (as discussed above). The use of two separate viability assays confirms increased cell killing; however, the limitations of these viability assays will be addressed.

Materials and Methods

Cells and Cell Culture

The PANC1 and Jurkat cells used in the study were purchased from American Type Culture Collection (Manassas, VA). PANC1 (human pancreatic carcinoma cell line) was chosen as an adherent cell line, whereas Jurkat (human T-lymphocyte cell line) was chosen as a nonadherent cell line. Cells were maintained at 5 % CO_2 and 37 °C in a humidified cell culture incubator. Dulbecco's modified Eagle medium (DMEM, American Type Culture Collection) supplemented with 10 % fetal calf serum, 2 mM L-glutamine and 2 % penicillin and streptomycin was used for culturing PANC1 cells, while Roswell Park Memorial Institute medium (RPMI, American Type Culture Collection) supplemented with 10 % fetal calf serum, 2 mM L-glutamine and 2 % penicillin and streptomycin was used for culturing Jurkat cells. Exponentially growing cells were used for all experiments.

Nanosecond Pulse Generator

A transmission line pulse generator was used to deliver electric pulses of 60-ns duration (as shown in Fig. 1) (Kolb et al. 2006). The pulse amplitude could be adjusted by varying the gap distance of any spark gap operated in open (atmospheric pressure) air.

Experimental Setup for Exposure to Ultrashort Electric Pulses

An optimal amount of T80 at 0.18 % was used for good dispersion, which had the least toxic effect on PANC1 and Jurkat cells (Sabuncu et al. 2010). Cell concentration was adjusted to 1×10^6/ml, and 900 µl of medium with suspended cells was used for experiments. To these cells, 200 µl of 1 % T80 was added, to bring the final concentration to 0.18 %; 400 µl of sample was exposed to 12 pulses of 30 kV/cm (60 ns, 3.2 joules) using electroporation cuvettes (BioSmith Biotech, San Diego, CA). After exposure, cell viability was analyzed using the trypan blue exclusion method (Tennant 1964) and WST-1 cell viability assay (Peskin and Winterbourn 2000) immediately after exposure to nsPEFs and after 8 h.

Cell Viability Assays

Cells exposed to nsPEFs were diluted at a concentration of 1:1 with 0.4 % trypan blue solution (Sigma, St. Louis, MO). Viable cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA). The trypan blue assay is based on uptake of the dye by the cell membrane of dead

cells; however, it is not able to differentiate between apoptotic and necrotic cells. For the WST-1 cell viability assay 100 µl of each sample (8 x 10^4 cells) was transferred into each well of a 96-well plate and 10 µl of WST-1 (Roche Applied Sciences, Indianapolis, IA) reagent was added to each well and incubated for 1 h. After incubation, the 96-well plate was read using a microplate reader at 450 nm. The WST-1 cell viability assay is based on cleavage of the water-soluble tetrazolium salt WST-1 into a soluble formazan dye by a complex cell surface mechanism of metabolically active and live cells. The optical density of the formazan dye is measured to obtain an estimate of live cells.

Cell Size Measurement Using Microscopy

PANC1 and Jurkat cells were viewed under a bright field microscope (Olympus, Center Valley, PA) before and after exposure (5 min) to nsPEFs. Images were captured using a DP71 CCD camera with DP controller software. Cell sizes were measured and analyzed using Image J software (NIH, Bethesda, MD).

Statistical Analysis

Cell viability assays were performed in triplicate, and results are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism™ (GraphPad Software, La Jolla, CA). Two-way ANOVA was used to demonstrate the significance among triplicate samples, and a two-tailed paired *t* test (95 % confidence interval) was used to detect significant differences between any two different samples. For all statistical analyses, $P < 0.05$ was considered significant.

Results

Viability of Jurkat and PANC1 cells was analyzed immediately after exposure to nsPEFs and 8 h postexposure. Cell viability after exposure was compared to cells that were not exposed to T80 or nsPEFs (control) and cells exposed to nsPEF only (control exposure).

The viability of Jurkat cells which were exposed to nsPEFs in the presence of 0.18 % T80 was assessed by trypan blue exclusion and WST-1 cell viability assay (Fig. 2). The results of trypan blue exclusion (Fig. 2a) and the WST-1 viability assay (Fig. 2b) immediately after exposure to nsPEFs demonstrate that there was no toxic effect of T80 on Jurkat cells compared to control cells. The trypan blue exclusion results also indicate that the cell viability decreased by 73 % in Jurkat cells that were exposed to nsPEFs in the presence of T80, which was highly significant compared to control exposed cells without T80 ($P < 0.01$, respectively), whereas WST-1 viability assay results of Jurkat cells (Fig. 2b) indicated that there was no significant difference in cell viability after exposure to T80 alone compared to control cells. Also, there was a significant decrease in cell viability by 68 % after exposure to nsPEFs in the presence of T80 compared to control exposed cells ($P < 0.01$). When exposed to nsPEFs for longer periods (8 h) Jurkat cells (Fig. 2c) demonstarted a significant effect of T80 alone on cell viability (without exposure to nsPEFs) ($P < 0.05$). These results also demonstrate that cell viability decreased significantly by 88 % in Jurkat cells that were exposed to nsPEFs in the presence of T80 compared to control exposed cells ($P < 0.001$). The WST-1 cell viability assay results of Jurkat cells 8 h after exposure to nsPEFs (Fig. 2d) demonstrate that there was no significant toxic effect of T80 on Jurkat cells compared to control cells in the absence of T80. It can be inferred from the results that there was a significant decrease in cell viability by 60 % in cells exposed to nsPEFs in the presence of T80 compared to control exposed cells ($P < 0.01$).

PANC1 cells that were exposed to nsPEFs in the presence of T80 were assessed just after exposure to nsPEFs (0 h) and after 8 h by trypan blue exclusion (Fig. 3a, c) and WST-1 cell viability assay (Fig. 3b, d). Trypan blue exclusion demonstrated that cell viability decreased significantly by 40 % in PANC1 cells which were exposed to nsPEFs in the presence of T80 compared to control exposed cells ($P < 0.01$). There was no significant toxic effect of T80 alone on PANC1 cells (Fig. 3a). WST-1 cell viability assay results demonstrate that there was a significant decrease in cell viability by 42 % in cells exposed to nsPEFs in the presence of T80 (Fig. 3b) compared to control exposed cells ($P < 0.01$). Analyzing 8 h after exposure to nsPEFs, trypan blue exclusion method results indicate that there was a highly significant decrease in cell viability by 95 % in cells exposed to nsPEFs in the presence of T80 (Fig. 3c) compared to control exposed cells ($P < 0.001$). The WST-1 cell viability results of PANC1 (Fig. 3d) indicate that there was no significant toxic effect of T80 alone. There was a very significant decrease in the cell viability by 50 % in cells that were exposed to nsPEFs in the presence of T80 compared to control exposed cells ($P < 0.001$).

We also measured the size of Jurkat and PANC1 cells (Fig. 4) prior to and postexposure to nsPEFs (5 min). We observed that the size of Jurkat cells (Fig. 4a) increased significantly in the presence of 0.18 % T80 ($P < 0.05$). There was a significant change in the size of Jurkat cells in the presence of 0.18 % T80 before compared to after exposure to nsPEFs ($P < 0.05$). There was no significant difference in PANC1 cell size postexposure to nsPEFs in the presence of 0.18 % T80 or cell culture medium (Fig. 4b).

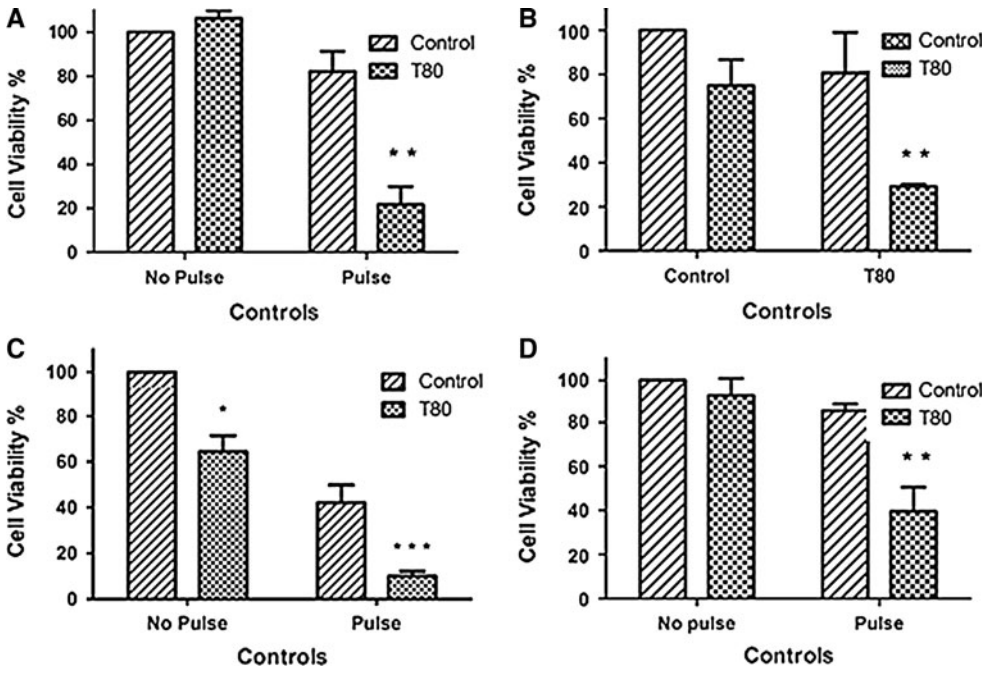

Fig. 2 Viability results of Jurkat cells after exposure to nsPEFs. **a** trypan blue assay results of Jurkat cells immeadiately after exposure to nsPEFs. **b** WST-1 cell viability assay results of Jurkat cells immeadiately after exposure to nsPEFs. **c** trypan blue assay results of Jurkat cells 8 h after exposure to nsPEF pulses. **d** WST-1 cell viability assay results of Jurkat cells 8 h after exposure to nsPEF pulses. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

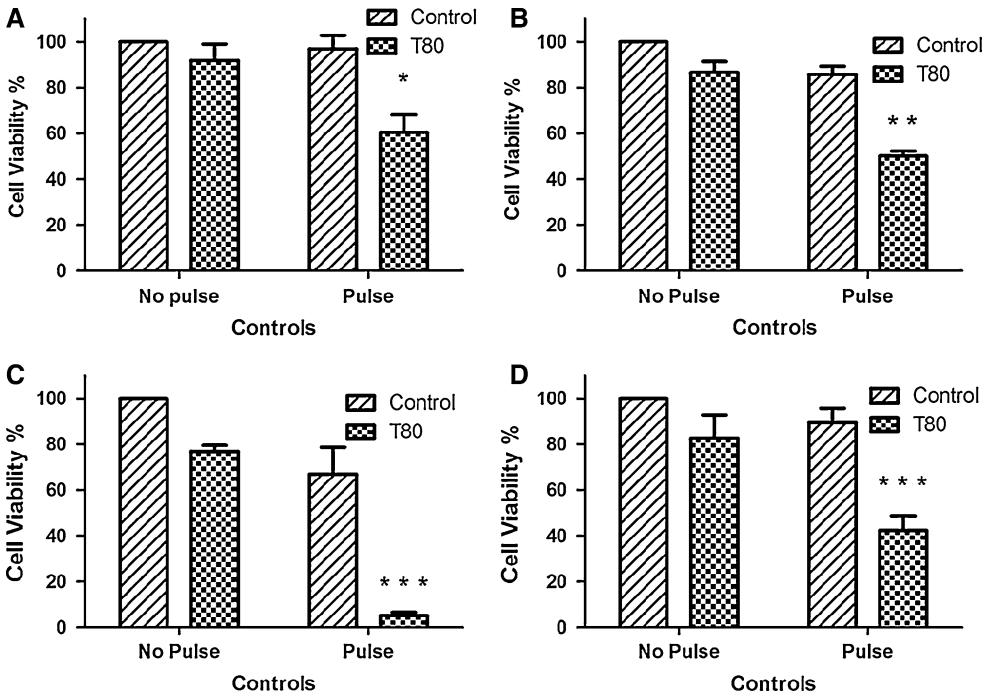

Fig. 3 Viability results of PANC1 cells (trypan blue assay and WST-1 cell viability assay) after exposure to nsPEFs. **a** trypan blue assay results of PANC1 cells immediately after exposure to nsPEFs. **b** WST-1 cell viability assay of PANC1 cells soon after exposure to nsPEFs. **c** trypan blue assay results of PANC1 cells 8 h after pulsing. **d** WST-1 cell viability assay results of PANC1 cells (8 h after pulsing). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

Discussion

Our results clearly indicate that there was no immediate significant toxic effect of 0.18 % T80 on the viability of Jurkat and PANC1 cells; however, after 8-h exposure Jurkat cells showed increased sensitivity. There was a significant reduction in the viability of PANC1 and Jurkat cells when exposed to nsPEFs in the presence of 0.18 % T80 (Figs. 2a, b, 3a, b). The decrease in cell viability was even higher 8 h after exposure to nsPEFs in the presence of 0.18 % T80 (Figs. 2c, d, 3c, d). It has been shown in the past that T80 has similar adjuvant activity with regard to drugs such as doxorubicin, ethoglucid, mitomycin-C and thiotepa in treating superficial bladder cancer (Parris et al. 1987) and increasing the antitumor efficiency of hyperthermia in treating B16 melanoma cells in BALB/C mice (Yaoqin et al. 1996). It has also been shown that T80 decreases drug resistance in actinomycin-D and daunomycin-resistant Chinese hamster cells (Riehm and Biedler 1972) and daunorubicin-resistant Ehrlich ascites cells

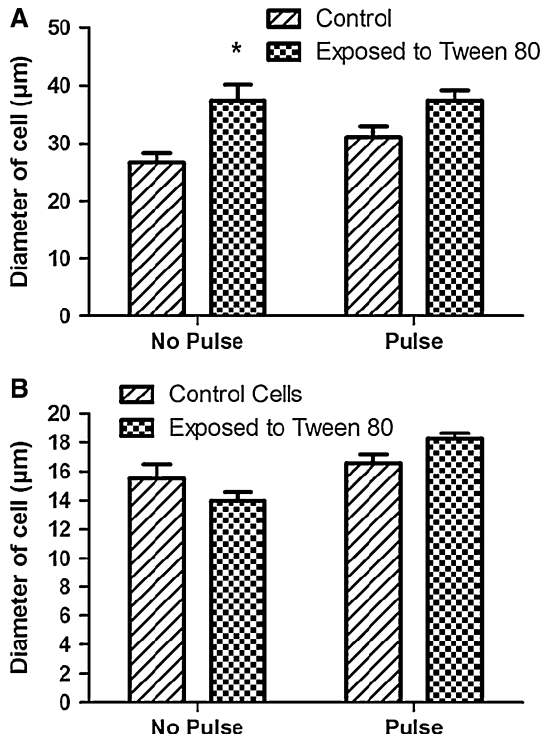

Fig. 4 Size variation in PANC1 and Jurkat cells in the presence of T80 and nsPEFs. **a** Change in Jurkat cell size when exposed to T80 alone, nsPEFs alone and both nsPEFs and T80. **b** Change in PANC1 cell size when exposed to T80 alone, nsPEFs only and both nsPEFs and T80

(Sehested et al. 1989). In all of these cases, T80 acted as a solubilizing agent to the plasma membrane of cells (Jones 1999) as well as in increasing membrane fluidity (Basrur et al. 1983; Chitnis et al. 1984; Tsujino et al. 1999; Coors et al. 2005). This increase in membrane fluidity could have significantly enhanced the poration (nano) effect of nsPEFs where stable poration is seen for minutes (Bowman et al. 2010). Better formulation of the possible effects of increased membrane fluidity in nsPEFs according to the reported effects in microsecond pulses is necessary (Kandušer et al. 2006). Another possible mechanism could be pore-induced changes in the osmotic pressure of cells, leading to an increase in the size of cells (Fig. 4), by the presence of T80. This effect, however, appeared to be cell type-specific, with Jurkat cells showing significant swelling. Jurkat cells are nonadherant and have reduced supporting cytoskeletal networks compared to adherent cells. The nsPEF-induced cytoskeletal damage (Stacey et al. 2011) along with T80-induced membrane fluidity may disrupt electrolyte balance, followed by water uptake, thus making Jurkat cells more susceptible to osmotic swelling.

Even though trypan blue and WST-1 cell viability assay results measured a significant killing effect of nsPEFs in the presence of 0.18 % T80, the difference in the cell viability results of these assays is primarily dependent on the cell component involved with the assay. The trypan blue exclusion assay is based on uptake of the dye by dead cells only, whereas the WST-1 cell viability assay is based on cleavage of WST-1 into a soluble formazan dye by mitochondrial dehydrogenase of metabolically active and live cells. T80 (0.5 %) (Wallace et al. 1968) has been implicated in mitochondrial and membrane biogenesis in yeast, a factor that was not investigated in these studies but which may influence both assay outcomes through different mechanisms. The plasma membrane is significantly affected after exposure to nsPEFs (André et al. 2010) measured by propidium iodide uptake across the cell membrane. The trypan blue assay is an assay based on the integrity of the plasma membrane. Therefore, with viability assays that may be compromised by T80 or membrane permeability, the results of WST-1 cell viability and trypan blue assays may be expected to differ from each other.

Conclusion

Based on our results, it can be concluded that T80 plays a major role in enhancing the killing effect of nsPEFs, which could be due to an adjuvant effect on PANC1 and Jurkat cells. We believe that T80 may increase the cell membrane permeability/fluidity, leading to increased fragility of the cell membrane. Further investigation is necessary to understand the mechanism of T80 as an adjuvant in enhancing the killing effect of nsPEFs. We also conclude that cell viability depends upon the protocol employed and may be indicative of different biological mechanisms that respective cell viability assays measure as a means to determine cell viability.

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