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Effect of Irreversible Electroporation on Three-Dimensional Cell Culture Model*

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Abstract—**Irreversible electroporation (IRE) is a new treatment to necrotize abnormal cells by high electric pulses. Electric potential difference over 1 V across the plasma membrane permanently permeabilizes the cell with keeping the extracellular matrix intact if the thermal damage due to the Joule heating effect is avoided. This is the largest advantage of the IRE compared to the other conventional treatment. However, since the IRE has just started to be used in clinical tests, it is important to predict the necrotized region that depends on pulse parameters and electrode arrangement. We therefore examined the numerical solution to the Laplace equation for the static electric field to predict the IRE-induced cell necrosis. Three-dimensionally (3-D) cultured cells in a tissue phantom were experimentally subjected to the electric pulses through a pair of puncture electrodes. The necrotized area was determined as a function of the pulse repetition and compared with the area that was estimated by the numerical analysis.**

I. Introduction

Electroporation has been widely used as an indispensable tool in basic research, plant breeding, biomedicine, and biotechnologies to introduce exogenous materials into cells [1]-[4]. When the cells are exposed to electric pulses with an appropriate condition, the permeability of the plasma membrane is increased, by which various kinds of materials such as proteins, DNA, organelles, pharmaceutical agents, and even whole cells can be finally transferred into the host cells. The electropermeabilized membrane is then resealed within a short time, which proceeds in a temperature-dependent manner. Since the electroporation needs to increase the transfection efficiency with minimizing the cell fatality, irreversible electroporation (IRE), which leads to cell death due to overloaded electric pulses, has been considered to be an undesirable side effect.

The side effect inducing permanent cell death has been recently attracting attention as a new method for tumor treatment. In a clinical application, a pulsed voltage of a few kilovolts is applied between electrodes that are inserted into abnormal tissues, and consequently, the cells surrounding the electrodes can be necrotized by the IRE [5]. The most significant advantage of the IRE is that it can destruct cells in the abnormal tissue without inducing any damage to the extracellular matrix (ECM), to the extent that the thermal damage due to the Joule heating is avoided. The conserved ECM contains a number of bioactive substances such as collagen, proteoglycan, and cell adhesion molecules. Additionally, it provides structural support and anchorage for cell proliferation. The IRE conserving the ECM intact is therefore a favorable treatment that promotes quick tissue regeneration after an ablation of the abnormal cells.

Successful IRE largely depends on a combination of pulse parameters and electrode configuration, which has been examined by numerical analyses as well as animal experiments. Rubinsky and his coworkers reported a series of studies to numerically estimate the distribution of electric fields and temperature rise during the IRE. For instance, Davalos et al. have employed the Laplace equation to calculate the electric potential generated in a tissue during the IRE and determined the effects of electrode geometry on the distribution of electric field and the volume that could be ablated [6]. Their numerical estimation showed that the IRE could ablate substantial volumes of tissue without causing any detrimental thermal effects. On the other hand, Al-Sakere et al. demonstrated through animal experiments that the prognosis after the tumor treatment depended on electric pulse parameters [7]. They treated mice aggressive cutaneous tumors using the IRE with different sets of pulse parameters, and thereby suggested that not only the electric field strength, but also the number of pulses and the total pulse duration affect the treatment outcome. To accomplish a complete ablation of the targeted tissue by the IRE with minimizing invasion, the precise estimation of the ablated volume depending on pulse parameters is needed before the treatment. However, no report has demonstrated that the numerical analyses based on the Laplace equation could predict the volume of the necrotized tissue although some studies in silico as well as in vivo indicated the usefulness of the IRE for tumor ablation.

The aim of this study was therefore to examine the application of the numerical solution for a prediction of the necrotized area by the IRE. An agarose gel containing 3-D cultured fibroblasts was used to mimic a physiological tissue and was subjected to electric pulses with different repetition. The necrotized area was determined as a function of the applied pulse condition and compared with the numerical solution to the Laplace equation.

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II. Materials and Methods

A. Three-Dimensional Cell Culture in a Tissue Phantom

Mouse embryonic fibroblast cell line NIH3T3-3 was obtained from ATCC (Manassas, VA). The cells were routinely maintained in α-modified minimum essential medium (α-MEM, Gibco BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin, Gibco BRL).

Low-melting-point agarose gel containing NIH3T3-3 cells was used as a tissue phantom for the electroporation experiment. Agarose powder (SeaPlaque Agarose, Lonza Rockland, Inc., Rockland, ME) was dissolved in distilled water at 80°C to the final concentration of 2.5 wt%, and then mixed with 10 times concentrated α-MEM solution and 200 mM HEPES at the volume ratio of 8:1:1. When the mixed sol was cooled down to approximately 37°C, NIH3T3-3 cells were added at a density of 2×10^5 cells/ml and used for the following experiment.

B. Electroporation Experiment

Experimental setup for the electroporation experiment is described in Fig. 1. The agarose sol containing NIH3T3-3 cells was poured into a plastic vessel (20 mm in inner diameter and 42 mm in depth), followed by inserting a pair of 1-mm dia. stainless steel electrodes at an interval of 5 mm. The length of 10 mm from the tip of the 20-mm-long electrodes was electrically conductive, and the rest of the lengh was insulated. The sol was gelated at 4°C for 20 min and further incubated in a CO_2 incubator at 37°C for 30 min.

Using a commercial square wave pulse generator (ECM830, Harvard Apparatus, Holliston, MA), direct current pulses of a voltage of 1000 V were applied between the electrodes. The duration and the interval of the pulses were set to be 10 μs and 100 ms, respectively. A sequence of 1, 15, 30, 45, 60, 75 or 90 pulses was chosen to evaluate effects of the pulse repetition on cell viability.

C. Cell Viability Assay

Following the electroporation, cell viability was determined using two fluorescent dyes: calcein AM forming green fluorescence in cytoplasm of alive cells and propidium iodide emitting red fluorescence at nuclei of dead cells.

The gel was removed from the plastic vessel and sliced perpendicularly to the electrode axis using an oscillating microtome (Vibroslice NVSL, World Precision Instruments, Sarasota, FL). The 1-mm thick section at the middle part of the electrode was stained with 2 μg/ml calcein AM (Nacalai Tesque, Kyoto, Japan) and 10 μg/ml PI (Molecular Probes, Carlsbad, CA) for 20 min. The double-stained section was observed with a fluorescent microscope (E600FN, Nikon, Tokyo, Japan) and fluorescent images were recorded.

The boundary between the red-fluorescent dead cells and the green-fluorescent intact cells was extracted using Photoshop (Adobe Systems, San Jose, CA), and then the area was calculated with ImageJ software.

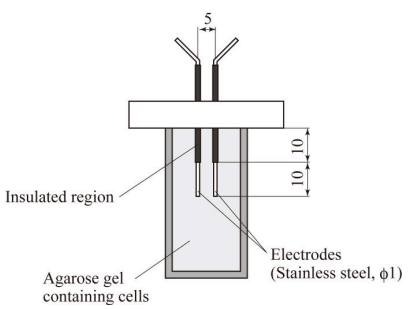

Figure 1. Electrodes inserted into tissue phantom. Units: mm.

D. Analytical Model and Equation

Configuration of the analytical model is described in two-dimensional Cartesian coordinate system in Fig. 2. A pair of electrodes with a diameter of d is placed in a tissue phantom, separated by a center-to-center distance of l. The voltage E is applied between the electrodes.

The distribution of electric potential within the tissue associated with an electric pulse can be determined by solving the Laplace equation:

$$\frac{\partial^2\phi}{\partial x^2}+\frac{\partial^2\phi}{\partial y^2}=0 \tag{1}$$

where ϕ is the electric potential. At boundaries where the tissue is in contact with electrodes,

$$\phi = E/2 \tag{2}$$

or

$$\phi = -E/2\,. \tag{3}$$

The electrical condition at the boundaries of the solution domain is assumed to be

$$\phi = 0 \tag{4}$$

to give a neutral base of the electric potential for calculation. The nondimensional form of (1) and the boundary conditions (2)-(4) are:

$$\frac{\partial^2\Phi}{\partial X^2}+\frac{\partial^2\Phi}{\partial Y^2}=0 \tag{5}$$

and

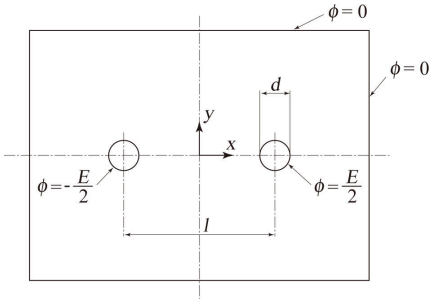

Figure 2. Physical model and coordinate system. Electrodes with a diameter of d is placed in a tissue phantom, separated by a distance of l. The electric potential ϕ is $-E/2$ and $E/2$ on each electrode surface when the voltage E is applied between the electrodes.

$$\Phi = 1 \quad \text{at positive electrode} \tag{6}$$

$$\Phi = -1 \quad \text{at negative electrode} \tag{7}$$

$$\Phi = 0 \quad \text{at the boundary of solution domain} \tag{8}$$

where

$$X = x / d \tag{9}$$

$$Y = y / d \tag{10}$$

$$\Phi = \phi / (E / 2) . \tag{11}$$

The numerical solution was obtained using a finite element analysis program Marc and pre/post-processing software Mentat (MSC Software Corp., Santa Ana, CA). Taking into account of the geometrical and electrical symmetry, only the first quadrant in Fig. 2 was defined as the solution domain and divided into 2298 triangle elements with 1199 nodes.

III. Results

Figure 3 shows a double-fluorescent micrograph of the sectioned tissue phantom after application of 45 pulses. Induction of PI-positive dead cells was observed around the electrodes. The necrotized region where PI-positive dead cells were distributed was manually extracted as shown in this figure, and its area A was calculated. Figure 4 shows the change of the necrotized area normalized by the area between the electrodes, $A/(d \times l)$, as a function of the number of applied electric pulses. While the cells survived a single shot of the pulse, the necrotized area increased almost linearly with the number of pulses, and seemed to reach a limit at 45 pulses. It however increased again with application of more than 75 pulses.

Numerical solution to (5) is shown in Figs. 5 and 6. Figure 5 shows the distribution of the electric potential along the X-axis, and Fig. 6 shows its gradient $\nabla\Phi$, *i.e.* the intensity of the electric field. The significant change in the electric potential was observed only at the vicinity of the electrode even at the region outside of both electrodes; $\Phi \approx 0.2$ at $X = 10$, indicating 80 % drop occurred within the region only seven time as far as the diameter of the electrode from the surface. The highest electric field was observed at the inside surface of the electrode facing to another electrode. It was approximately 1.5 times higher than that at the outside surface.

The intensity of the electric field is the most important index for necrosis of cells. Since the critical voltage that causes the breakdown of the cell membrane is believed to be approximately 1 V at room temperature [8], the voltage of at least 2 V may be required for a cell to induce irreversible damage. Neglecting the possible distortion of the electric field, we estimate the critical electric field necessary for the membrane breakdown by

$$\nabla\Phi_{\text{cri}} = \frac{1 \times 2/(E/2)}{d_{\text{cell}}/d} \tag{12}$$

where d_{cell} is the diameter of a cell. Using the mean diameter of the NIH3T3-3 cells, 16 μm, in the tissue phantom that was preliminarily measured by a microscope, and the potential

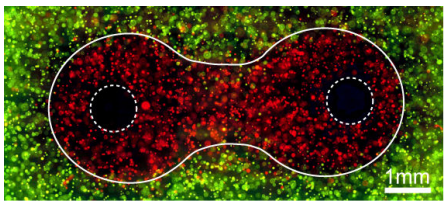

Figure 3. Live (green) and dead (red) cell distribution in the sectioned tissue phantom after an application of 45 pulses. Dashed circles describe the position where the electrodes located. The boundary between live and dead cells (white line) was extracted to calculate the nocrotized area.

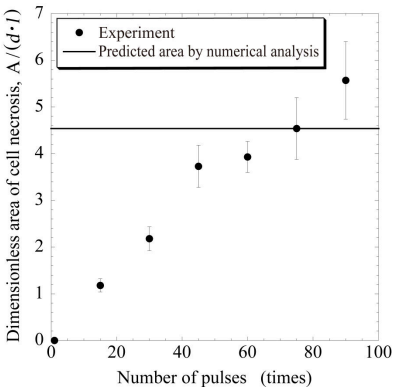

Figure 4. Change of the necrotized area as a function of the number of the electric pulses.

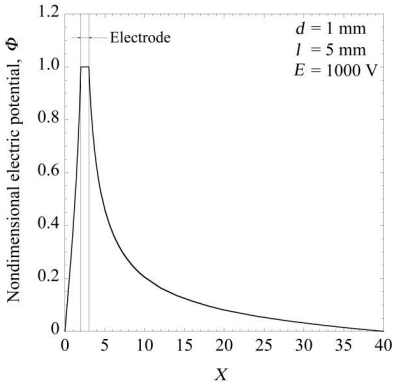

Figure 5. Electric potential distribution on the axis of X-dimension. Nondimensional electric potential Φ is defined as $\phi/(E/2)$.

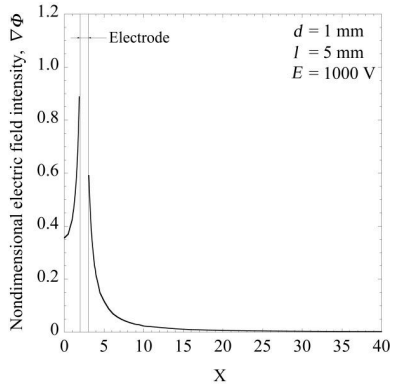

Figure 6. Electric field intensity on the axis of X-dimension. Nondimensional electric field intensity $\nabla\Phi$ is calculated by $\sqrt{(\partial\Phi/\partial X)^2 + (\partial\Phi/\partial Y)^2}$.

difference 1000 V with the electrode diameter 1 mm, we obtained $\nabla\Phi_{cri} = 0.25$ for the present case. The present criterion therefore indicates that the cells in the region where $\nabla\Phi > 0.25$ could be irreversibly destructed. Figure 7 indicates the contour of the electric field obtained by the numerical analysis. The gourd-shaped contour for $\nabla\Phi = 0.25$ is similar to the necrotized area shown in Fig. 4. In addition, the normalized area of $\nabla\Phi > 0.25$ was 4.54, which was only slightly higher than the that after application of 45 to 75 pulses in Fig. 4.

IV. Discussion

The cell-necrotized area predicted by the numerical solution based on the Laplace equation was compared to the cell viability that was experimentally determined using 3-D cultured cells in a tissue phantom. Although only a 2-D analysis was conducted in the present study, distribution of the electric potential in the cross section at the middle of the electrode is expected to be the same as that obtained by the 2-D analysis because of likely symmetry of the electric field at top and bottom.

Cells are believed to be permanently destructed by the electric potential difference of 1 V across the cell membrane at room temperature [8]. However, our experiment demonstrated that an application of only a single pulse was not enough for inducing the cell necrosis. The necrotized area increased with the number of pulses, indicating that multiple pulses were required to fully destruct cells even if the electric field exceeded a threshold for breakdown of the cell membrane. This is probably because that the cell membrane is not charged up to the voltage sufficient for the breakdown by a pulse shorter than a few tens of microseconds. Once the breakdown voltage has been reached, the membrane destruction occurs within submicroseconds [9]. The charging time depends on the potential difference, the cell radius, the capacity of the membrane, and the electrical resistivity of the surrounding solution. Since the charging time is shorter for higher potential difference, the electric charge would be completed first in the cells exposed to the larger potential gradient near the electrodes. The necrotized area could then expands gradually to the periphery with pulse repetition.

After the cell-necrotized area reached a plateau after application of 45 to 75 pulses, it increased again with the pulse repetition, which is probably attributed to the prolonged electroporation. The electropermeabilized membrane can generally recover its integrity by a resealing process. As far as the resealing process completely repairs the permeabilized membrane between pulses, the cell viability is maintained. However, the prolonged application of the electric pulses would accumulate the incomplete structure and assembly of the membrane, and finally leads to irreversible defects. A collapse of the balance between the electropermeabilization and resealing process possibly induces the gradual expansion of the cell-necrotized area with further application of electric pulses.

Although the numerical solution to the Laplace equation with the threshold of 1 V for the membrane breakdown agreed well with the plateau in the cell-necrotized area observed in the experiment, it did not provide a successful

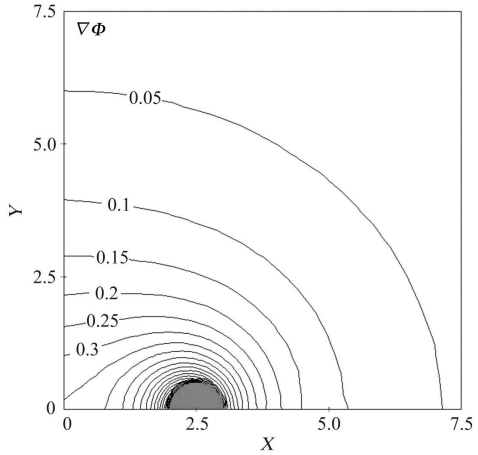

Figure 7. Contour of electric field around the electrode. Nondimensional electric field intensity $\nabla\Phi$ is calculated by $\sqrt{(\partial\Phi/\partial X)^2 + (\partial\Phi/\partial Y)^2}$. Gray semicircle indicates the position where the electrode located.

prediction of the necrotized area as a function of the number of pulses. Even if the potential difference of 1 V generally provides a criterion for the breakdown of the membrane, the necrosis of cells is a result of a dynamic process, which could not be fully described as a static problem. The combination of pulse width, intervals and the number of repetition does have considerable effects on the membrane destruction and resealing processes. The distribution of the electrical properties and its change during continuation of pulse repetition are another factors that should be taken into account for detailed estimation of the outcome of the IRE.

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