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The Effect of Electrical Discharges in the Cell Media on Their Viability and DNA Damage and Comparison with the Effect of X-Rays

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ABSTRACT: This study measures the viability of human breast cancer cells and the effect on DNA exposed to electrically generated plasmas produced directly in the growth media, and these findings are compared with the results of x-ray exposure. The effect of increasing plasma exposure is found to follow the same pattern as that from 160-keVp x-ray exposure to both DNA damage and cell viability. These results add support to the idea that plasma and x-ray–induced chemistry in liquids are similar, and may help to define and quantify the concept of dose in plasma applications in medicine.

KEY WORDS: discharge in liquid, cancer cells, viability, DNA damage, dose

I. INTRODUCTION

The application of plasmas for medical purposes has recently become a reality.[1] Most of the plasmas currently used are created in flowing gas, usually helium or argon, at atmospheric pressure. Here the discharge is created directly in cell-containing Dulbecco's modified Eagle's medium (DMEM), which is a conducting solution. We investigate the response of the MDA-MB-231 human breast cancer cell line to plasma exposure using a clonogenic assay to determine the viability of the treated cells and a γ-H2AX assay to investigate induced DNA damage in the cell nucleus (e.g., DNA strand breaks). Results from plasma exposure are compared with the effects of x-ray irradiation of the same cells because emerging evidence shows that much of the chemistry in plasma–liquid interactions is similar to that ultimately produced in radiolysis. This finding may be significant because there is a very developed approach to defining the dose delivered in x-ray treatment (measured in Gray [Gy], where 1 Gy = 1 J/1 kg) and the relative biological effectiveness of that dose.

II. METHODOLOGY

A. Overview

Plated cells were exposed to either radiation or plasma at various doses or to a number of plasma pulses, respectively. After exposure to either radiation or plasma, the cells were

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assayed for cell viability (i.e., the ability to form viable colonies) or DNA damage. Experiments were performed in quadrature and the standard error was used as a measure of the uncertainty. In all cases, control samples were sham irradiated or sham exposed to plasma (i.e., all other steps were performed on the samples in an identical fashion except the radiation or plasma source was not turned on). Details of the procedures are described below.

B. Plasma Apparatus

Fig. 1 shows the experimental apparatus used. The apparatus is a modified version of a system used to study plasma formation in saline solution, which was previously described in detail.[2,3] Briefly, the powered electrode is a 0.5-mm diameter tungsten wire that extends 0.5 mm beyond an encasing and supporting 6-mm diameter glass capillary. The return electrode is a 10-mm inner diameter, 1-mm-thick, coaxial, stainless steel, hollow cylinder with thin holes above the liquid level to prevent gas and liquid trapping. Both electrodes were in contact with the cell-containing media. The inner electrode was fully immersed in the media, with the lower face approximately 0.5 mm above the glass substrate onto which cells had previously been plated.

Fig. 1 shows the experimental arrangement used for the γ-H2AX assay, in which the cells are adhered to a glass microscope coverslip placed in a 35-mm diameter petri dish that was filled with 6 ml media.

For the cell viability assay, the petri dish and glass plate were replaced with one well of a Nunc six-well plate. The cells were seeded on the bottom of the well. This setup was used because reseeding of the cells is required for analysis and it is essential that none of the cells be excluded from treatment. To achieve this, the diameter of the return electrode was increased so that it matched the 35-mm well diameter. The distance of the powered electrode to the bottom of the wells was maintained at 0.5 mm via micrometer adjustment.

C. X-Ray Source

Cells were irradiated with 160-kVp x-rays at a dose rate of 4.37 Gy min^{-1} using a Faxitron CP-160 x-ray generator (Faxitron X-ray Corporation, Lincolnshire, IL) with the samples placed at one of the standard target-sample distances of 12.7 cm. This x-ray irradiator has a tungsten anode inclined at 22° to the electron beam and a 0.8-mm-thick beryllium and 0.5-mm-thick copper filters. The x-ray spectrum that it produces is predominantly due to bremsstrahlung radiation, which results in a broad peak ranging from 30 keV up to 160 keV, on top of which lie the characteristic x-ray lines of tungsten. The exact details of the x-ray spectrum are not important in this study, because the biological response does not vary significantly over this energy range.

D. Cell Preparation

An adherent human breast cancer cell line (MDA-MB-231; American Type Culture Collection, Manassas, VA) was used. Cells were grown in DMEM. Before exposure, we

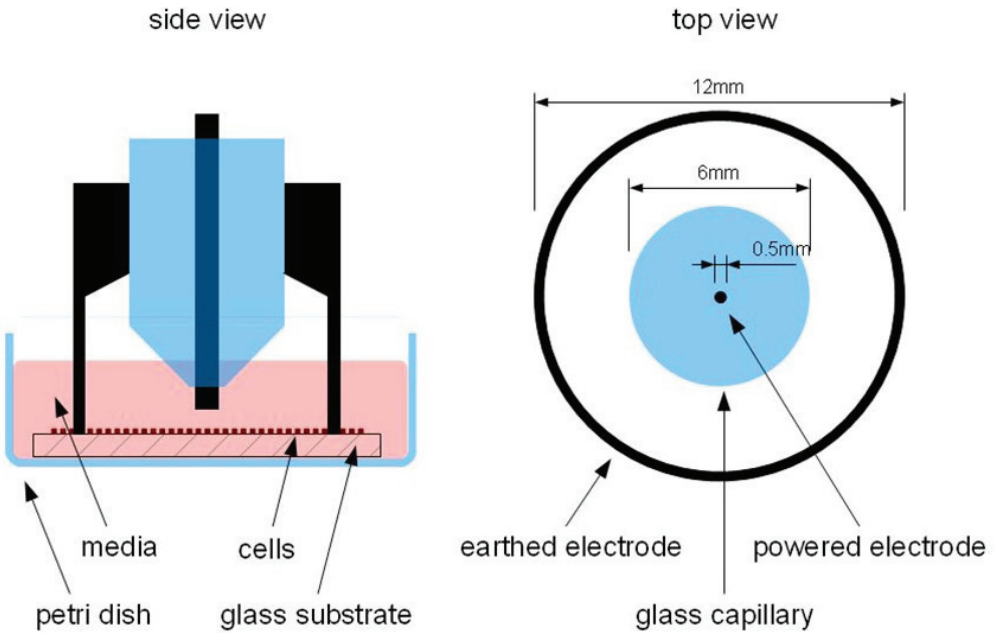

FIG. 1: A schematic diagram of the plasma apparatus used for the cell treatment with cut-through views of the coaxial setup in side view (left) and top view (right).

added the following to the cell growth media: antibiotics penicillin and streptomycin, for prevention of bacterial growth; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), a pH buffering solution. The cells were incubated for at least 24 hours to allow them to adhere to 18 × 18 mm^2 glass microscope coverslips and to grow to an approximately 70% confluence level. These microscope slips were then placed in petri dishes 35 mm in diameter filled with 6 ml DMEM, resulting in a filling height of about 6 mm.

E. Cell Exposure

In both experimental setups, the discharge electrical properties were kept the same. The plasma was produced by applying a potential difference of 350 V between two electrodes that were immersed in the conducting cell culture medium (DMEM). The current draw through the liquid created a gaseous layer around the central electrode. Once the vapor isolates the electrode from the conducting liquid, an electric field is present across the liquid-vapor gap and, if this field is sufficiently high, plasma is created.[2,3] The voltage pulse length was 0.3 ms and the plasma persisted for approximately 140 μs. This sequence occurred for each voltage pulse, which was applied at a frequency of 2 Hz

Fig. 2 shows a typical current waveform in cell growth media (DMEM) with the phases of gas layer nucleation and the formation of continuous discharge indicated. The

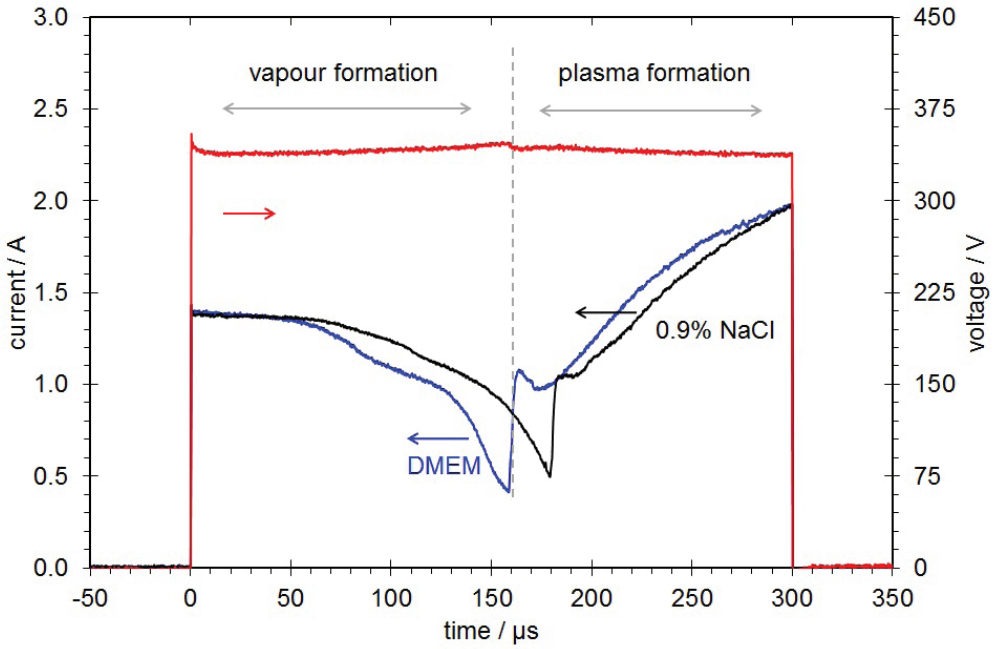

FIG. 2: A typical current-voltage characteristic in DMEM compared with the current characteristic of 0.9% w/v NaCl in deionized water.

current waveforms of the two liquids used here, 1× phosphate-buffered saline (PBS; with sodium azide) and DMEM (complete with fetal bovine serum, penicillin, streptomycin, and HEPES), are very similar to each other and to those in normal saline solution of 0.9% NaCl. The characteristic and sequential vapor phase and plasma formation are described in detail by Schaper *et al.*[2,3]

F. DNA Damage

The γ-H2AX assay[5] was used to investigate induced DNA damage in the cells nuclei. After plasma treatment, the cells were left in the cell culture media for 1 h before the media were removed and the cells were washed with PBS. The cells were then fixed for 10 min at 37°C with a 1:1 methanol-acetone mixture that was at –18°C when added. The fixing solution was then removed and the cells were again washed with PBS before preserving them in PBS with 0.09% sodium azide. The cells were prepared for staining by removing the PBS and sodium azide, washing with PBS, and adding 0.5% Triton X-100 in PBS at 4°C to make the cell membranes permeable. This buffer was then removed and the cells were washed in PBS and a blocking buffer consisting of 0.2% skim milk, 5% horse serum, and 0.1% Triton X-100 in PBS was added and left on the cells for 2 h at room temperature to block unspecific binding sites. After 2 h, the blocking buffer was removed and 100 µl of the 10,000:1 in blocking buffer–diluted primary antibody mouse

monoclonal IgG1 clone JBW (α-γ-H2AX antibody anti-phospo-H2AX [Ser139]) was added on top of the cells, which were then covered by parafilm and left in the dark at room temperature for 2 h. After 2 h, the leftover liquid was removed and the cells were washed with PBS containing 0.1% Trypsin.

The cell nucleus was additionally stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain by adding 1 ml of DAPI in PBS solution at a concentration of about 5 μg/ml to the cells, which were incubated at room temperature in the dark for 5 min and then washed with PBS. After staining, the coverslips with the cells were mounted with DAPI free mounting medium (Vectashield; Vector Labs, Burlingame, CA) onto microscope slides (upside down, so that the cells were not exposed to air anymore) and imaged with a fluorescence microscope. Figs. 3 and 4 present examples showing the overlay of the two different fluorescent signals. The blue fluorescence is due to DAPI and indicates the position of the cell nuclei, whereas the bright green foci within the nucleus indicate the places where there is a DNA damage site undergoing repair. The more diffuse green background signal is typical and is most likely caused by either residue or minor interaction of the secondary antibody with the cytoplasma.

Figs. 3 and 4 show that the plasma-treated cells have far more DNA damage sites than the untreated cells. Foci from >100 cells were counted in each case using images like these for subsequent quantitative analysis.

G. Cell Viability Assay

A clonogenic assay was used to assess the effect of the plasma treatment on cell viability. After exposure to the plasma, the cells were left in the growth media for an hour before the treated media was removed and the cells were washed with PBS three times. A solution of 0.1% Trypsin in PBS was then added to detach the cells and the samples were left in an incubator at 37°C until the cells were completely detached (about 15 min). Culture media were then added to neutralize the Trypsin. The detached cells were then

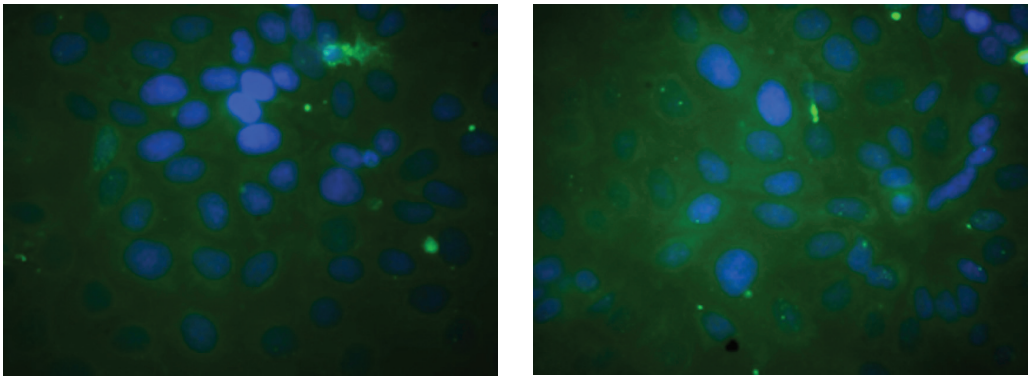

FIG. 3: Untreated cells with their nuclei (blue) and corresponding γ-H2AX signal (bright green foci). Each focus in a nucleus indicates a DNA damage site.

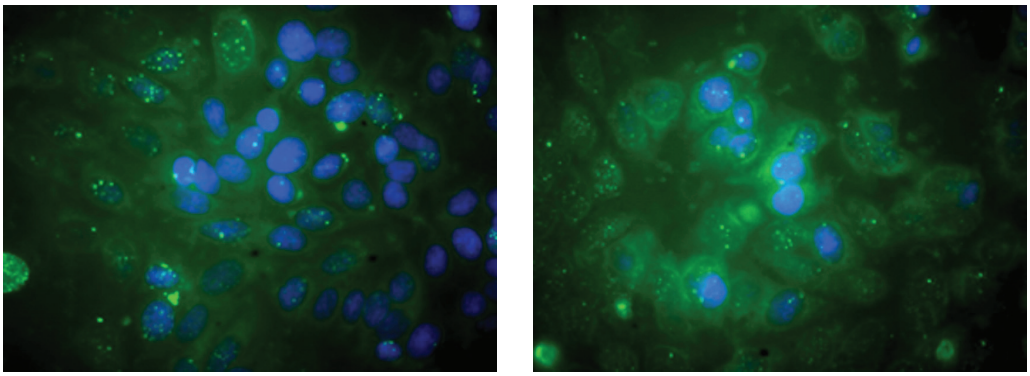

FIG. 4: Cells treated by 40 pulses with nuclei (blue) and DNA damage (green spots within nucleus) labeled.

passed over a cell counter and a specific number of cells, depending on the number of plasma pulses or radiation dose used, were extracted and resuspended for cultivation and colony growth. The cells were stored in an incubator for 10 d to allow colonies to form, after which they were washed with PBS twice. The colonies were then covered with a solution of 0.1% Crystal Violet and 2% ethanol diluted in deionized water for 30 min, after which the solution was removed and the cells were rinsed with tap water. The blue-violet–colored dye Crystal Violet is absorbed by the cells and leads to color contrast of the cell colonies to the background.[4] Cell colonies were then counted in the usual fashion using an automated ColCount (Oxford Optronix, Abingdon, Oxford, UK) colony counter and were also randomly checked via a manual count. Colony counts for the sham-irradiated samples were used to determine the plating efficiency, from which the survival fraction was determined for each exposure.

III. RESULTS AND DISCUSSION

A. Analysis of DNA Damage

For analysis of DNA damage, according to the protocol described in section II.F, the foci occurring in 150 cell nuclei were counted for the applied doses. In this case, the cells were exposed to up to 40 plasma pulses or 1 Gy of x-rays. For example, Fig. 4 shows plots of the number of cells with a corresponding number of foci in an unexposed control as well as exposures to 10 and 40 plasma pulses and to 1 Gy of 160 keVp x-rays.

The control shows that there is some naturally occurring DNA damage in cells. In Fig. 5, we fit the number of the 150 cells with a given number of foci indicating DNA damage to Poisson distribution curves. It is known[11] from experiments with different cell treatment methods that the damage distribution in untreated cells follows the Poisson distribution function, as found here. It is also known that x-ray exposure to doses of up to 5 Gy also induces a Poisson damage distribution. Our results at 1 Gy are

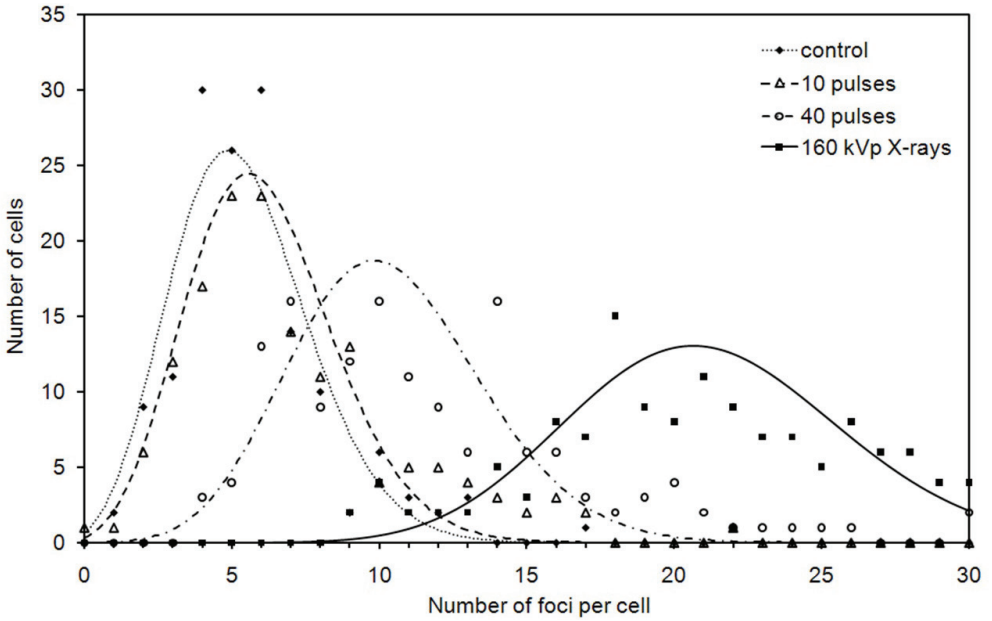

FIG. 5: DNA damage to the cells measured using the γ-H2AX assay and the corresponding Poisson function fits for the control (black diamonds and dashed line), cells exposed to 10 plasma pulses (white triangles and dashed line), 40 plasma pulses (white circles and dasheddotted line), and 1 Gy of x-ray treatment (black squares and solid line).

consistent with this. From Fig. 5, it is clear that this is also true for the cells exposed to plasma treatment.

Fig. 5 also shows that the DNA damage found in cells increases with plasma exposure, which is indicated by the shift of the distribution function maximum toward a higher number of foci per cell. This behavior is reflected in the plot of the average number of damaged foci per cell shown in Fig. 6, which also shows that there is a linear dependence on the average number of foci per cell to the number of applied plasma pulses. The measured average number of foci per cell for 1 Gy x-ray irradiation is also shown. This indicates that approximately 100 plasma pulses would cause similar DNA damage in the cancer cells used here.

This relationship is also reinforced by Fig. 7, in which the axes for the number of pulses and the x-ray dose have been shifted so that the location on the top *x*-axis for 1 Gy of radiation and the bottom for 100 plasma pulses coincide.

B. Cell Viability

Fig. 7 plots the surviving fractions of cell colonies as a function of exposure to up to 150 plasma pulses and up to 4 Gy of x-rays. The error is calculated from the standard deviation of four samples for the plasma-treated samples, whereas the results

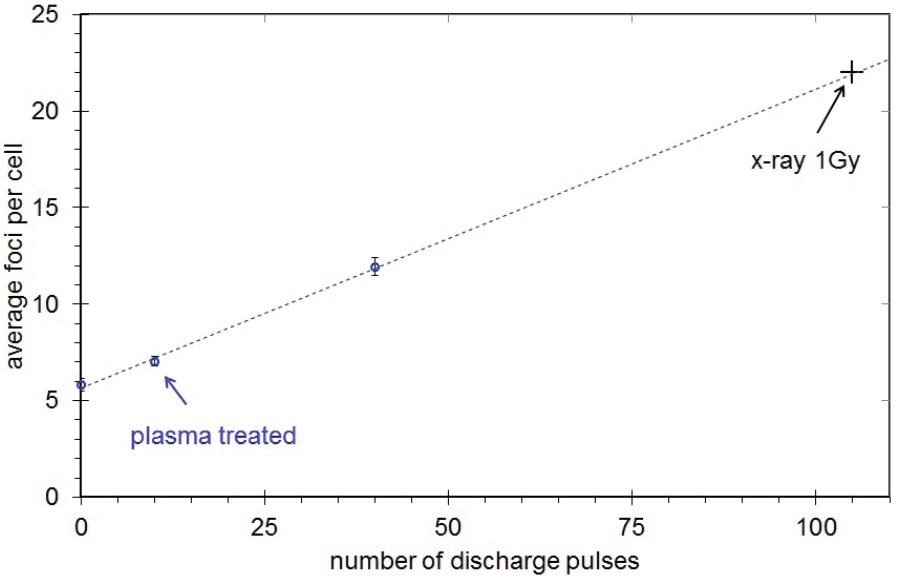

FIG. 6: The average number of DNA damage foci per cell versus number of plasma pulses compared with the x-ray pulse equivalent.

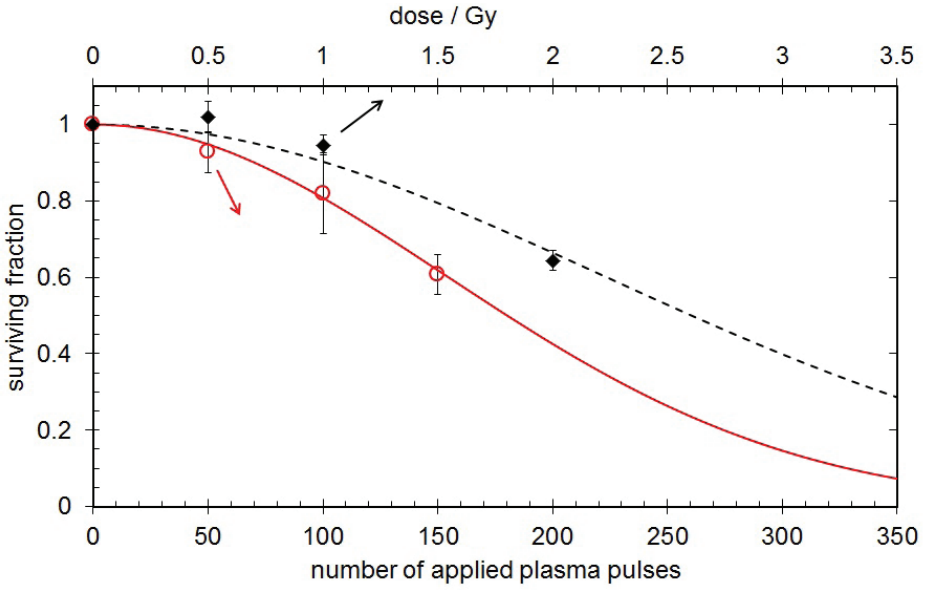

FIG. 7: The surviving fraction dependence on the number of plasma pulses (white circles and bottom axis) and x-ray dose (black filled diamonds and top axis).

for the x-ray–treated cells and their standard deviations are calculated on the basis of 10 samples.

For cell irradiation by x-rays, it is known that the resulting surviving fractions, sf(D), are described by linear quadratic exponential curves[6] that for the treatment single dose conditions used here are best described by Eq. 1:

$$sf(D) = \exp(-D \times (\alpha + \beta D)) \quad (1)$$

where D is the dose, α is the sensitivity to direct so-called "single track" damage, and β is the sensitivity to successive or "multitrack" damage due to interaction of two or more particles.[7] Generally, this relation, ignoring repopulation and cell replication, proves to be valid for doses up to at least 5 Gy.[8,9]

For the plasma-treated samples, the same assumption of a linear quadratic dose response has been applied and fits to both the plasma and x-ray data sets using a least-squares routine. These fits yield values of $\alpha = 0 \pm 4 \times 10^{-2}$ and $\beta = 0.010 \pm 0.001$ for the x-ray data and $\alpha = 0 \pm 4.7 \times 10^{-2}$ and $\beta = 2.18 \pm 0.37 \times 10^{-5}$ for the plasma data. If the data are interpreted according to the x-ray model, then damage is caused by two separate events in both cases.

IV. CONCLUSIONS

Electrical plasmas produced in media containing human breast cancer cells are found to reduce the subsequent viability of the cells and to create damage to the DNA in the cell nucleus. The effect of increasing plasma exposure is found to follow the same pattern as that from 160-keVp x-ray exposure. This adds further to support to the idea that plasma- and x-ray–induced chemistry in liquids are similar and this may assist in helping to define and quantify the concept of dose in plasma applications in medicine.

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