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Micronucleus formation induced by dielectric barrier discharge plasma exposure in brain cancer cells

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Induction of micronucleus formation (cytogenetic damage) in brain cancer cells upon exposure of dielectric barrier discharge plasma has been investigated. We have investigated the influence of exposure and incubation times on T98G brain cancer cells by using growth kinetic, clonogenic, and micronucleus formation assay. We found that micronucleus formation rate directly depends on the plasma exposure time. It is also shown that colony formation capacity of cells has been inhibited by the treatment of plasma at all doses. Cell death and micronucleus formation are shown to be significantly elevated by 120 and 240 s exposure of dielectric barrier discharge plasma. © *2012 American Institute of Physics*. [doi:10.1063/1.3687172]

Nonthermal plasma is emerging as a tool for the treatment of living tissues for biological and medical purposes.[1–20] Plasma contains free charges (electron, ions), free radicals, excited molecules, and UV photon and generates transient electric fields. Several previous studies have demonstrated the efficacy of sterilization by plasma application, and more recently, some studies have shown an antitumor effect of plasma *in vitro* on few type of cancer.[17–20] Current research mainly focuses on the non-thermal effects of dielectric barrier discharge (DBD) plasmas: aim to inducing a specific response or chemical modification by generating active species that are either produced in the plasma or in the tissue brought into contact with plasma.[14] Nonthermal plasmas also have least effect on the surrounding (healthy) tissue, but allow efficient biological activity against microbes and cell within a minute. After the *in-vitro* studies on various animal and human cells, the effect of various cold atmospheric plasmas on genetic material or chromosome damage remained only a question of time. So in this study we focused on genotoxicity analyses of DBD plasma on brain cancer cells. For this specific purpose, we studied micronucleus formation by plasma exposure on T98G cells, which is a human brain cancer cell line.

The observation that chromosome damage could be caused by exposure either to ionizing radiation or carcinogenic chemicals has been among the reliable evidence that physical and chemical agents can cause major alterations to the genetic material of eukaryotic cells.[21] It has been proposed independently by Schmid[22] and Heddle[23] that an alternative and simpler approach to assess chromosome damage *in vivo* is to measure micronuclei, also known as Howell–Jolly bodies to haematologists, in rapidly dividing cell populations such as the bone-marrow and cancer. The micronucleus assay in cancer biology is now one of the best established *in vivo*/*in vitro* cytogenetic assays in the field of genetic toxicology. It is evident that the *in vitro* micronucleus assay has evolved into a robust assay for genetic damage with applications in ecotoxicology, nutrition, radiation sensitivity testing for cancer risk assessment and optimisation of radiotherapy, biomonitoring of human populations, and important testing of pharmaceuticals and agrichemicals.[24]

The effect of DBD plasma on micronucleus formation in brain cancer cells has not been described by scientific community yet. Therefore, major purpose of our present study is to investigate the effect of DBD plasma on the genetic material for different types of cell lines. Here, we are showing the growth kinetics, clonogenicity, and micronucleus formation by DBD plasma in T98G brain cancer cell line.

Figure 1 shows a nonthermal coplanar DBD plasma for the treatment of T98G cells. The culture cells have been treated by 3 mm distances away from the plasma source up to 240 s. Cell suspension has been made in media layer which is 2 mm in thickness. Cells have been treated by the DBD plasma for 30–240 s under electrical discharge power of about 18 W (2.2 kV, 11 mA, and phase angle 0.7 radian) with 100 V input voltage. For high-voltage power supply, a commercial transformer for neon light operated at 60 Hz is used. The upper electrode is made of ITO paste electrode, and down electrode facing the sample is made of stainless steel mesh. They are separated by 3 mm-thick glass and tightly sealed by insulating paste. The diameter of electrodes is 9 cm, which is designed for 10 cm petri-dish. Power supply is by Slidacs, a neon trance invertor. Working temperature of plasma source is in the range of 24–32 °C at the time of treatment. The diameter of metal mesh for DBD plasma is 80 mm, the thickness of mesh is 1 mm, the separation distance between two adjacent metal grids is 2 mm, and the width of silver electrode is 3 μm, which is coated on the glass, whose thickness is 1.8 mm, by screen printing method. Figure 2 shows the growth kinetics of T98G cells. Cells proliferation kinetics have been studied at 24, 48, 72 h after plasma treatment, following trypsinization and counting total cells per plate by using a trypan blue dye and hemocytometer. The data obtained by growth kinetics assay showed that our plasma have inhibitory effect on the growth of T98G cells in exposure and incubation time dependent manners. It is noted the cells exposed to 30 and 60 s plasma treatment show less effects than those with 120 and 240 s treatment. Maximum effect is shown by 240 s plasma exposure; it

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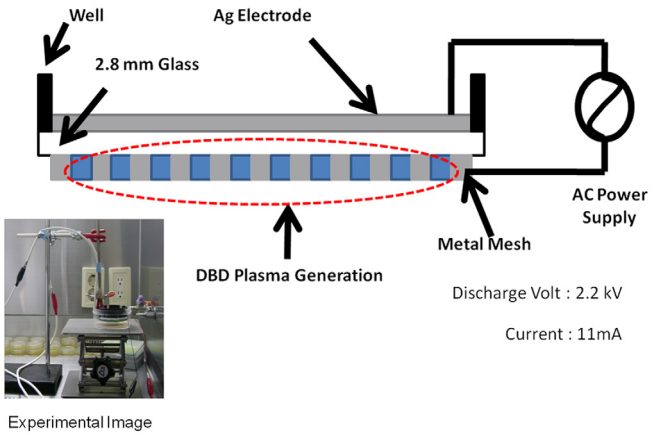

FIG. 1. (Color online) Schematic diagram of our dielectric barrier discharge plasma system consisting mainly of a high-voltage power supply, electrodes, and dielectrics.

inhibits the growth of cells up to 80% at 48 and 72 h after treatment and its viability range is 19%-20%. In the case of 30, 60, and 120 s exposure, we found that up to 70% cells death and their viability is 58%, 66%, and 73%, respectively, for 72 h after treatment. Cell morphology analysis has revealed that the shape and size of viable cells are also affected by plasma exposure. We have found in Fig. 2 the remarkable differences in shape and size of treated cells at 2 and 48 h after 240 s plasma treatment.

Figure 3 shows the effect of DBD palsma on the colony forming capacity and survival of exponentially growing T98G cells. We have used the clonogenic assay for confirming our growth inhibition results by plasma exposure. Clonogenic assay or colony formation assay is an *in vitro* assay based on the ability of a single cell to grow into a colony. The assay essentially tests every cell in the population for its ability to undergo unlimited division.[25] Only a fraction of seeded cells retains the capacity to produce colonies after plasma treatment. In this assay, 200 treated cells have been seeded out in appropriate dilutions to form colonies in 12 days. Colonies have been fixed with methanol, stained with 1% crystal violet, and counted for survival fraction curve. It is observed in the Fig. 3 that the survival fraction of T98G cells has been drastically decreased by the treatment time in clonogenic assay. Plasma treatments enhance cell death and also inhibit colony formation capability in the T98G cell population as plasma treatment time increases. T98G cells with plasma treatment for respective different exposure time have shown the decline in the survival fraction, as evidenced by the reduction in the number of colonies formed (as shown in the right hand side of Fig. 3). Even for 30 and 60 s plasma exposure, the significant decline in colony survival has been observed and their ranges are up to 27%-28% and 31%-33%, respectively. However, significant drastic decline in the survival fraction could be observed after exposure to 120 and 240 s plasma treatment and their survival percentage is almost 0 (zero). It shows that these treatments are significantly inhibiting the colony formation capabilities of brain cancer cells at all doses (plasma exposure) and utmost clonogenic inhibitory effect, which we found after plasma exposure of 120–240 s.

Overall by growth kinetics and clonogenic assay results, we can conclude that plasma exposure has significant inhibitory effect on T98G cells growth and may have genotoxic effect on cells. So it becomes necessary to do a piece of work related to genotoxicity.

In the recent years, the *in vitro* micronucleus assay has become an attractive tool for measuring genotoxicity by any physical and chemical agents, because of its capacity to detect clastogenic and aneugenic events, simplicity of scoring, accuracy, multipotentiality, and wide applicability in different cell types.[26,27] The purpose of the micronucleus assay is to detect those chemical and physical agents which modify chromosome structure and segregation in cells. For measuring micronucleus, we have treated T98G cells by plasma under similar conditions as we have used in earlier experiments for growth kinetics and clonogenic assay. After exposure by DBD plasma, cell cultures are grown for 24 and 48 h to allow chromosomal damage to lead to the formation of micronuclei in bi- or multinucleated interphase cells. Harvested and Hoechst

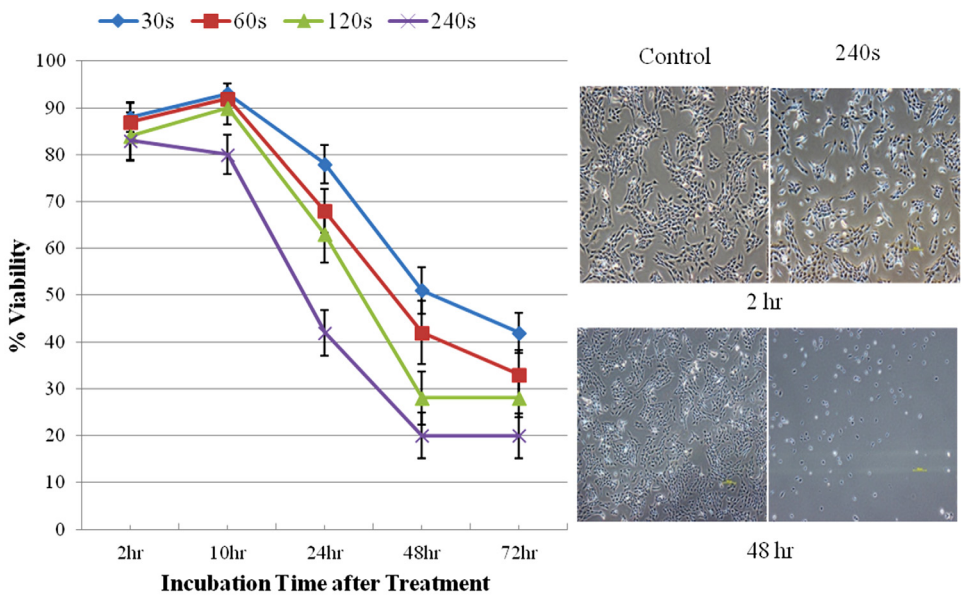

FIG. 2. (Color online) Growth kinetics of T98G cells at 24, 48, and 72 h after treatment by DBD plasma. T98G cells were treated with 30-240 min plasma irradiation. Untreated cells are taken as control, and the data represents mean ± SEM, n = 3.

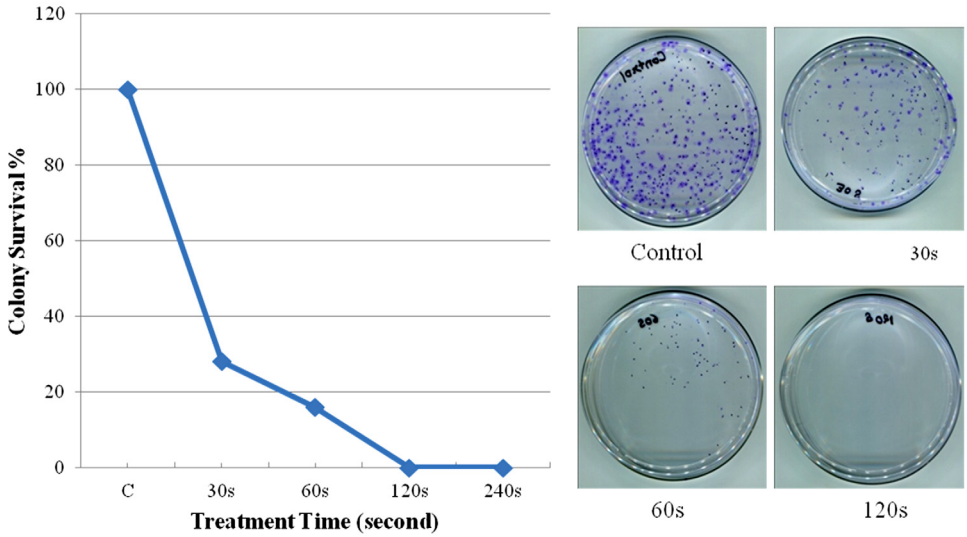

FIG. 3. (Color online) Effect of DBD plasma on colony forming capacity and survival of exponentially growing T98G cell lines studied by macro colony assay. The data represents mean, n = 3.

33342 stained interphase cells are then analysed microscopically for the presence of micronuclei.

Mitotic death (linked to cytogenetic damage) and interphase death (apoptosis) together account for the cytotoxicity of many physicochemical agents, although the relative contributions of the two death processes vary among the type of damaging agent. To investigate the plasma-induced cytogenetic damage by using Hoechst-33342, we have studied the plasma treatment-induced micronuclei formation in T98G cell line. Since cell proliferation may influences treatment-induced micronuclei expression, the data from kinetic studies up to 48 h post treatment have been analyzed. Figure 4 shows the micronucleus frequency at 24 and 48 h for T98G brain cancer cell after plasma treatment by 30–240 s. The frequency of untreated cells with micronuclei has been in the range of 3.6%-4.2%, and treatment with 30 s plasma exposure does not induce a significant level of micronuclei formation in T98G cells, but induces micronucleus 5.4% and 6.54%, respectively, at 24 and 48 h. However, micronuclei frequency increased from 3.6% (control) to 11.01%, 17.12%, and 22.6% for 60, 120, and 240 s plasma irradiated cells, respectively, at 24 h of culture. However, it is increased further at 48 h of culture. It is shown for the plasma irradiation for 60, 120, and 240 s that micronuclei frequency has been further enhanced from 4.2% (control) to 12.12%, 18.11%, and 26.64%, respectively, at 48 h of culture after treatment. Micronucleus shown in plasma irradiated cells is formed by DNA strand breaks generated during the faulty excision repair process. The remaining unsealed DNA leads to the formation of micronuclei in subsequent mitosis, and cells with micronuclei are found to be associated with the loss of reproductive capacity.

Finally, we can conclude that plasma could have the broad exposure dosage ranges of activity against the human T98G brain cancer cells. Plasma treatment is shown to be toxic on T98G cells and least toxic on normal human embryonic kidney (HEK) cells. The 70% of brain normal cells are neuronal cells and the rest of cells are different kinds of normal cells. The HEK cell line resembles developing neuron and neuronal stem cells and also mentioned as good model

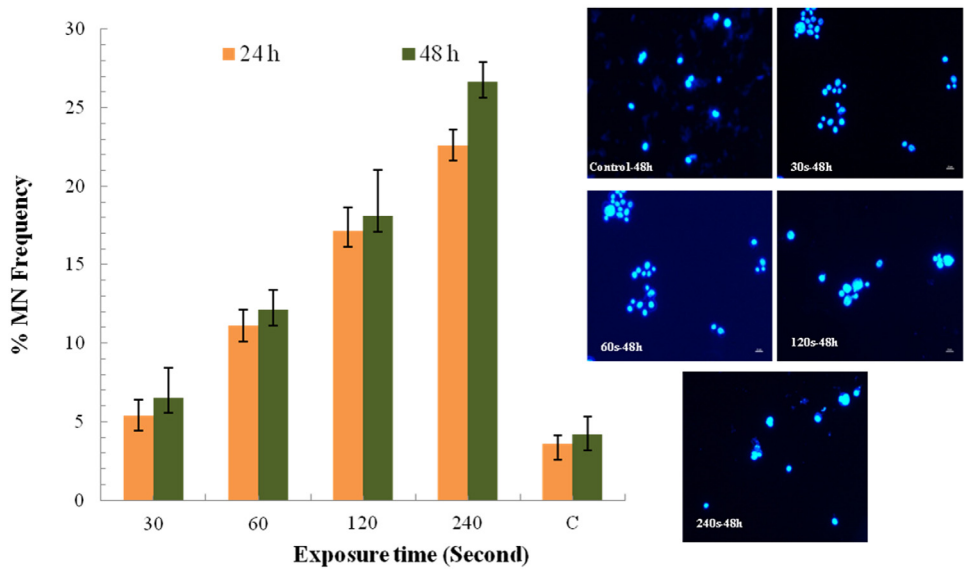

FIG. 4. (Color online) Comparison between micronucleus frequencies at 24 and 48 h in human T98G cells treated with 30-240 s DBD plasma, whereas "control" is the micronuclei frequency of untreated cells. The data represents mean ± SEM, n = 3.

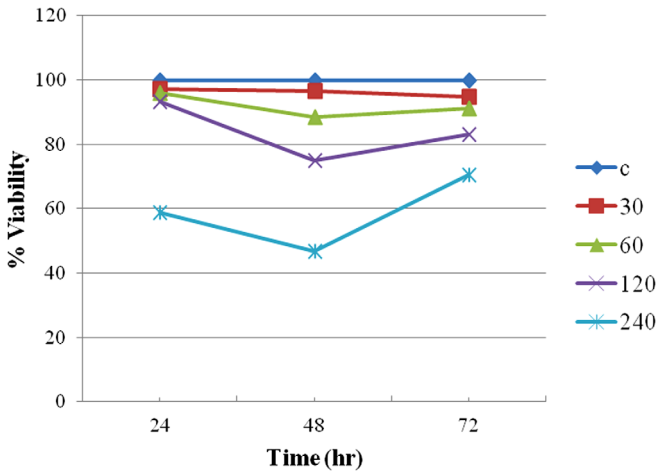

FIG. 5. (Color online) % Viability of HEK cells at 24, 48, and 72 h after treatment by DBD plasma for 30, 60, 120, and 240 s. The data represents mean, n = 3.

for neuroscience studies.[28] From this background, we have used HEK normal cells as a model for brain normal cells in this study. Figure 5 shows the viability range of 30, 60, and 120 s plasma treated HEK cells, which is higher than 75% at all incubation time (24, 48, and 72 h) after treatment. Plasma treatment may be regarded as class of anticancer agent with sensitization capability. The present results of the antiproliferative studies by plasma treatment clearly demonstrate that plasma treatments sensitize T98G cells, by increasing both the mitotic (linked to cytogenetic damage) and loss of clonogenicity. Enhanced chromosomal aberrations in treated cells are mainly responsible for mitotic death of cells. Micronuclei formation is, therefore, one of the good indices to measure the residual DNA damage and loss of reproductive ability of cells. The enhanced cell death due to plasma exposure has been also accompanied by a significant increase (2-3 folds at all exposure) in micronuclei formation in T98G brain cancer cell line. However, the plasma may also induce a higher rate of faulty mitosis in damaged cells, which could partly contribute to the enhanced micronuclei observed.

These studies indicate that DBD plasma able to induce micronucleus formation and behaves as genotoxic physiochemical agent. Further researches on fate of cell with micronucleus induced by plasma exposure will be going on and reported in due time for the investigation of effect on normal human cells.

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