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Corrigendum

Corrigendum to "Effect of jet plasma on T98G human brain cancer cells" [Curr. Appl. Phys. 13 (2012) 176–180]

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The author regrets that in the above article an error occurred in the references. There is a switch between references [20] and [21]

The corrected version of the article is as follows,

Abstract

Nonthermal plasma (an ionized gas) is emerging as a novel tool for the treatment of living tissues for biological and medical purposes. In this study we describe the effects of a nonthermal air jet plasma on the T98G brain cancer cell line. The results of this study reveal that the jet plasma inhibits brain cancer cell growth efficiently with the loss of clongenicity in the cells. The main goal of this study is to induce cell death in T98G cancer cells by the toxic effect of jet plasma.

Keywords: Jet plasma, T98G cell, Brain cancer

1. Introduction

Nonthermal plasma (an ionized gas) is an emerging novel tool for the treatment of living tissues for biological and medical purposes [1–6]. Plasma contains free charges (electron, ions), free radicals, excited molecules, photons (UV) and generates a transient electric field. Several studies have demonstrated the efficacy of sterilization by atmospheric nonthermal plasma [7–19]. Moreover, the recent studies have also shown an antitumor effect of plasma *in vitro* on a few types of cancer [2–6]. Current research mainly focuses on the nonthermal effects of plasma on tissue, applications below the threshold of thermal damage that aim at inducing a specific response or chemical modification by generating active radical species that are either produced in the plasma or in the tissue brought into contact with plasma [18]. Nonthermal plasmas have shown the least effect on the surrounding (healthy) tissue, by allowing efficient biological activity within a minute. After previous *in vitro* studies on various animal and human cells, the application of cold atmospheric plasma on live living tissues found to remain for a matter of time. Overall, the anticancer properties of cold plasma having the broad spectrum of medical applications have paved the way for analysis on human living tissues and patients.

Atmospheric pressure (AP) nonthermal plasma jets have also been recently gained much attention in biomedical society [20–24]. The effects of AP nonthermal plasma jet on T98G brain cancer cells (gliomas) have not been described by the scientific community. In the present investigation we have developed a nonthermal air jet plasma to assess the anticancer effect on gliomas. In this study, we have shown the effect of jet plasma on T98G brain cancer cells in presence of an air gas flow.

2. Materials and method

2.1. Plasma treatment

Fig. 1 displays the plasma jet system consisting mainly of a high voltage power supply, electrodes and dielectrics. We used nonthermal jet plasma for the treatment of cells in the presence of an air gas flow. A commercial transformer for the neon light at 60 Hz is used for the high-voltage power supply. The primary voltage of the high voltage transformer is regulated by a voltage controller. The inner electrode is a typical injection needle made of stainless steel with an inner diameter of 1.2 mm and a thickness of 0.2 mm, it is tightly covered by a quartz tube with an outer diameter of 7 mm. The outer electrode is also fabricated from stainless steel and it is centrally perforated with a hole of 1 mm, through which the plasma jet is ejected into the surrounding ambient air. Porous alumina with a diameter of 12 mm and a length of 17 mm is machined to be in tight contact with the inner and outer electrodes. The discharge gap distance is adjusted to 2 mm between the inner and outer electrode. Gas is injected into the injection needle and then ejected through the 1 mm hole in the outer electrode via the porous alumina. The porous alumina used in this work is approximately 30 vol % porosity with an average pore diameter of 100 μm. Air is used as the feeding gas, the flow rate is controlled by an analog controller. Once gas is introduced through the inner electrode and high-voltage ac power is applied, a discharge is fired into the porous alumina between the electrodes and a long plasma jet reaching lengths of up to several centimeters is ejected into the open air. Cells were treated with jet plasma for 30 s, 60 s, 120 s and 240 s under an electrical discharge power of about 18 W (2.2 kV and 11 mA) with a 100 V input voltage. The driving frequency is 20 kHz and flow rate of air gas is kept to 1 L per minute. The working

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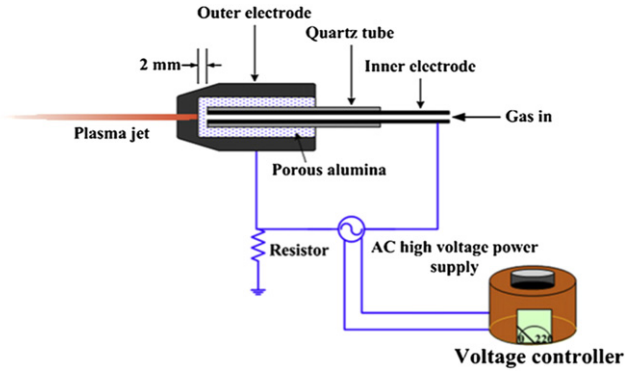

Fig. 1. A plasma jet system consisting mainly of a high-voltage power supply, electrodes and dielectrics. Distance between the cell media and outer electrode was kept to 5–6 mm during exposure. Air gas flow rate in jet plasma is 1 L/min for treatment. Cells are in suspension state in DMEM cell culture media. Density of cells in suspension for plasma treatment is 10^3 cells/ml.

temperature of the plasma source is in the range of 26–36 °C at the time of treatment.

2.2. Human cell culture

The T98G brain cancer cells used in the present study were purchased from KCLB (Korean Cell Line Bank). We cultured these cell lines in 75 cm^2 culture flasks (Corning, USA) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% glutamine, 1% penicillin (100 IU/ml) and streptomycin (100 mg/ml) (all from Hyclone, USA) or by the distributor instructions. All cultures were maintained at 37 °C, 95% relative humidity and 5% CO_2. Prior to each test, the cells were harvested using trypsin–ethylenediamine tetraacetic acid (EDTA)–PBS solution and diluted by upto 5×10^5 cells/ml for experiment. Stock cultures were passaged every third day after harvesting the cells with 0.05% trypsin and seeding 8×10^3 cells/cm^2 in tissue culture flasks to maintain the cells in the exponential phase. All experiments were carried out on exponentially growing cells. The cells must be in the exponential phase for experiments because cell growth is slow or reduced in the lag and stationary phase. If the cells reach the stationary phase they make clumps which is not recommended for growing cells in culture or for experiments. The cell suspensions were seeded into 100 mm cell culture plates (Corning, USA) at 100 μl/plate, and incubated for approximately 20–24 h before the experiments in order to reach confluency. The plates were treated with 0.01% poly-D-lysine solution (Sigma–Aldrich, USA). In the present study we performed MTT assay, growth kinetic assay, analysis of cell morphology and clonogenic assay of the cells treated by jet plasma.

2.3. Cell growth kinetics assay

Cells were seeded at 7000–10,000 cells/cm^2 on the cell culture plate and their proliferation kinetics were studied at different incubation times (24, 48 and 72 h) after plasma treatment, following trypsinization and counting the total cells per plate microscopically using a trypan blue dye and hemocytometer.

2.4. MTT assay

Cells were seeded in culture plates at a concentration of 2–4×10^5 cells/plate in 5 ml of complete media and incubated for 12 h at 37 °C in a 5% CO_2 atmosphere to allow for cell adhesion. Cells were treated with plasma for 30, 60, 120 and 240 s. A control group without plasma treatment was included in each assay. After incubation times of 24, 48 and 72 h, plasma exposures to cells was performed at different doses, each plate was carefully rinsed with 1 ml phosphate buffered saline (PBS). Viability was assessed using MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide). 250 μL MTT solutions (5 mg mL^{-1} PBS) along with 4 ml of fresh and complete media were added to each plate and the plates were incubated for 3 h. Following incubation, the medium were removed and the purple formazan precipitates in each well were sterilized in 2 ml DMSO (Dimethyl sulphoxide). All assays were performed as three independent sets of tests. Absorbance was measured using a microplate reader at 540 nm and the results are expressed as a % viability, which is directly proportional to the metabolic active cell number [25–27]. Percentage (%) viability was calculated as:

$$\%\text{Viability} = \text{Optical Density in sample well} / \text{Optical Density in control well} \times 100$$

2.5. Clonogenic survival assay

Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. A colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo "unlimited" division. Only a fraction of seeded cells retains the capacity to produce colonies before or after treatment, cells were seeded out in appropriate dilutions to form colonies in 10 days. After harvesting with 0.05% trypsin, 150–400 (depending on the treatment) cells were plated 10–14 h before plasma treatment in DMEM at 37 °C [28]. Cultured cells were treated with plasma for 30, 60, 120 and 240 s. After the treatment, cells were incubated in the dark under a humidified, 5% CO_2 atmosphere at 37 °C for 10 days to allow colony formation. In vitro cell growth is recommended to be performed in the dark because the metabolic process of cells is much more effective in the dark compared to in light. Colonies were fixed with methanol and stained with 1% crystal violet. Colonies of more than 50 cells were counted and the % survival was calculated. Clonogenic survival curves were constructed by average value from three independent experiments. We used the clonogenic assay as an *in vitro* surrogate assay of tumorigenicity. The media used allows for three-dimensional growth of cell colonies that more closely resembles a tumor than a monolayer.

2.6. Statistical analysis

Data is expressed as a mean ± SD. Statistical analysis was performed by student *T*-tests. Results were considered significant when $P < 0.05$.

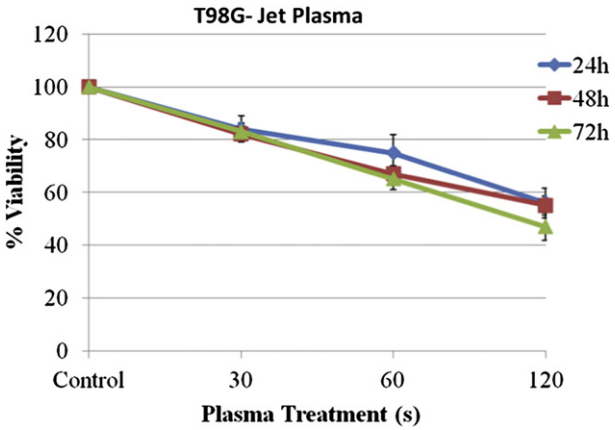

Fig. 2. Percent growth of T98G cells at 24, 48 and 72 h after treatment by jet plasma. T98G cells were treated with plasma irradiation of 30–240 s. Heamocytometer is used for counting cells after treatment and results are shown as the % viability. Untreated cells were used as a control and all values given as a mean (±SE) of three independent experiments, $n = 3$. All values are statically significant, $P < 0.01$.

3. Results and discussion

3.1. Growth inhibitory effect of jet plasma on T98G cells

Fig. 2 shows the growth kinetics of the T98G cells. Data obtained by growth kinetics assay showed that the jet plasma has an inhibitory effect on T98G cells that is dependent on exposure time. The culture cells were treated at 6 mm from the plasma source for 30–240 s. Cell suspensions were made in media layers of 3 mm thickness. As a note, the cells exposed to 30 s and 60 s, plasma treatments show less effect than those exposed to a 120 s treatment. The maximum effect was seen after 120 s plasma exposure, by inhibiting the cell growth upto 45% and 53% at 48 and 72 h respectively after the treatment with viability range of 47–55%. Whereas in case of a 60 s exposure, we found that upto 35% cells were died and their viability was 75%, 67% and 65% at 24, 48 and 72 h respectively after treatment. Cell morphology analysis revealed that the growth pattern of the T98G cells was also affected by jet plasma exposure. Fig. 3 shows the differences in growth pattern of the treated cells population, compared to the untreated control. There was a remarkable difference in the growth patterns of the 120 s treated cell population and the untreated control. Overall, plasma inhibits the growth of T98G cells in a dose dependent manner and the plasma treatment for 120 s has the maximum inhibitory effect as compared to other treatments. These experiments indicate that plasma inhibits T98G cancer cell growth which may be due to either growth arrest or cell death. We also performed MTT assay and clonogenic assay to confirm cell death in T98G cells by plasma.

3.2. MTT assay (mitochondrial activity)

We performed a MTT assay to after growth kinetic assay for measuring cell viability. An attempt was also made to correlate the growth kinetics of T98G cells. MTT was reduced by viable cells to a colored formazan product and has been used for chemo and radiosensitivity testing. The 3-(4,5-dimethylthiazol-2-yl)-2,5-

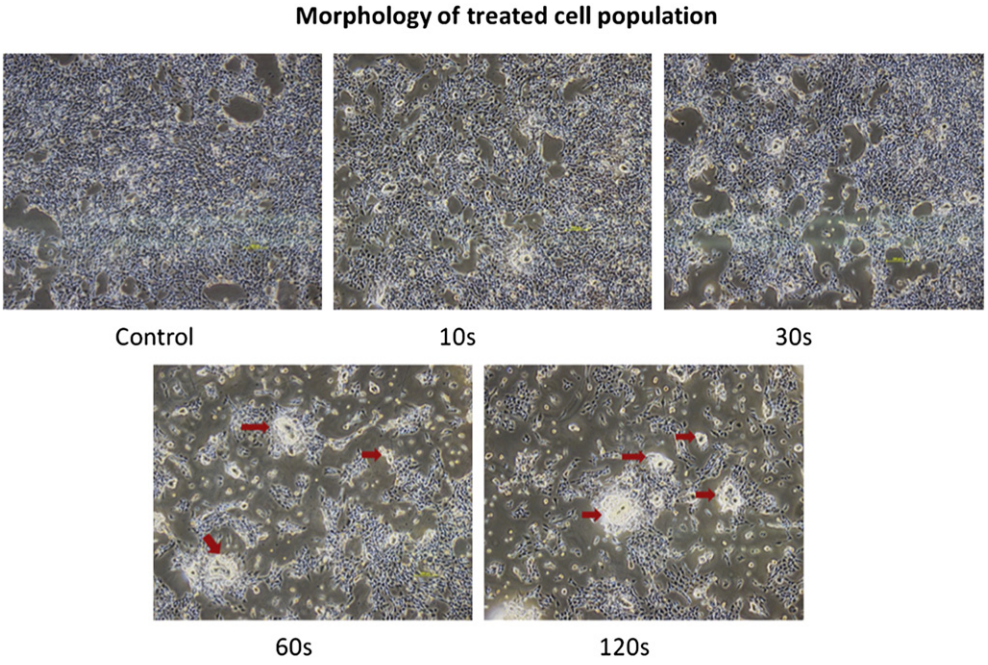

Fig. 3. The morphology of T98G cells after treatment by plasma. Comparison is shown between cells treated with plasma and the untreated control. Arrow is showing difference in growth pattern of T98G cell population after treatment (with plasma) when compared with untreated (without plasma) control. All pictures are taken at 24 h after treatment by using a NIKON inverted microscope.

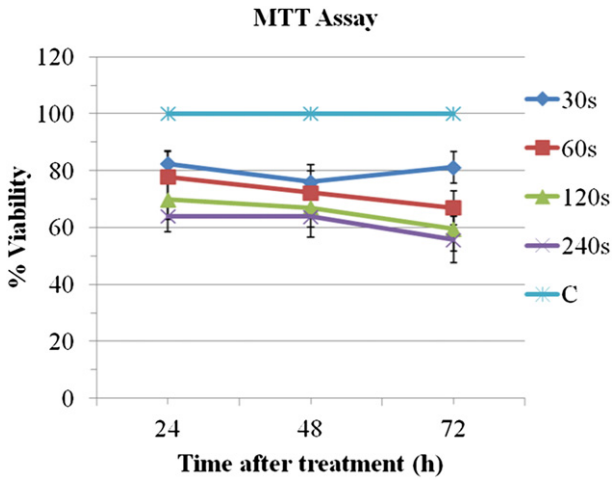

Fig. 4. Effect of jet plasma on T98G cells at 24, 48 and 72 h after treatment by MTT assay. T98G cells were treated with 30–240 s plasma irradiation with an air gas flow. Results are shown as the % viability. All values are compared with an untreated control and given as a mean (±SE) of three independent experiments, $n = 3$. All values are significant, $P < 0.05$.

diphenyltetrazolium bromide (MTT) cell proliferation assay has been widely accepted as a reliable way to measure the cell proliferation rate, and conversely when metabolic events lead to cell death (apoptosis or necrosis). Fig. 4 shows the viability of T98G cells treated for 30, 60, 120 and 240 s with jet plasma. As seen in Fig. 4, plasma treatment (30–240 s) of T98G brain cancer cell line resulted in a dose dependent decrease in absorption at 540 nm indicting decreased reduction of MTT to its formazan product by mitochondrial dehydrogenase. In the MTT assay, T98G cells treated with jet plasma for 120 s show a significant decrease in mitochondrial activity while their cell viability was 69%, 67% and 59% at 24, 48 and 72 h, respectively. Whereas a 240 s jet plasma exposure results in a drastic decrease in mitochondrial dehydrogenase activity with a 37–45% viability of cells. Data obtained by MTT assay shows that the plasma has an inhibitory effect on the growth of T98G cells dependent on exposure and incubation time. There was a remarkable difference between the 30 s exposure and other treatments, 30 s treatment having less inhibitory effect as compared to the other plasma doses. Overall, the MTT assay also explains the dose dependent decrease in the growth of T98G cells and shows that the 240 s plasma treatment has the maximum inhibitory effect. However, a cytostatic effect was observed upto 72 h in the case of 30 s plasma treatment. Overall, these results clearly demonstrate that the plasma sensitizes T98G cells in a dose dependent manner, which may be by increasing the induced mitotic (linked to cytogenetic damage) or interphase (apoptosis) death.

3.3. *Effect of jet plasma on clonogenicity of T98G cells*

Fig. 5 shows the effect of the jet plasma on the colony forming capacity of T98G cells. We used a clonogenic assay to confirm the colony formation inhibition results due to plasma exposure. Fig. 5 shows that the survival percent of T98G cells was drastically decreased and this directly depends on exposure time. This *in vitro* clonogenicity method was recognized as a valid surrogate assay for tumor growth *in vivo*. As plasma exposure increases, the Plasma treatments enhance cell death and inhibit the colony formation capability of the T98G cell population. Plasma treatment for the respective different exposure times results in the decline of the survival percentage of T98G cells, which can be seen by the reduction in the number of colonies formed, as shown on the right side of Fig. 5. Even for 30 and 60 s plasma exposures, a decline in colony survival of up to 79% and 73% respectively was observed. However, a significant, drastic decline in the survival percentage

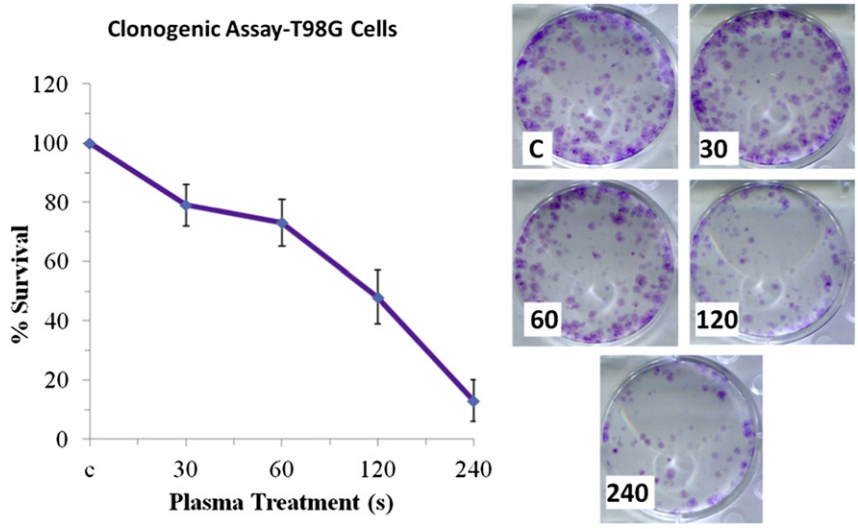

Fig. 5. The effect of jet plasma on clonogenic capacity and survival of exponentially growing T98G cell lines studied by clonogenic assay. The data presented shows mean values from three independent observations, $n = 3$.

was observed after exposure to 120 and 240 s plasma treatments with survival percentages of 48% and 13%, respectively. Overall, the reduction of colonies after 20, 60, 120 and 240 s plasma treatments was 21%, 27%, 52% and 87% respectively. This shows that these treatments are inhibiting the colony formation capabilities of T98G cells at all doses and also indicates that plasma treatment causes cell death as reflected by decreased clonogenicity. The clonogenicity results can be interpreted as an indication that brain cancer cells have increased tumorigenic capabilities and plasma is effective in reducing the tumorigenic potential of the human brain cancer cell line. The present study clearly demonstrates the inhibitory effects on T98G cells, by increasing both the cell death and loss of clonogenicity in treated populations. This suggests an inhibition of the repair processes and enhanced damage fixation within the cells. Loss of clonogenicity following the induction of growth inhibition could arise either due to mitotic death and/or due to apoptosis in T98G brain cancer cells. Based on these changes in the brain cancer cell lines, we conclude that AP jet plasma affects T98G brain cancer cells which justify future preclinical studies in animal models of brain cancer.

4. Conclusion

Plasma treatment found to be toxic for T98G cells. The present results of anticancer studies on jet plasma treatment clearly demonstrate that jet plasma treatments sensitize T98G cells, by increasing both the cell death and loss of clonogenicity. Overall, according to the growth kinetics, MTT and clonogenic assay results, we can conclude that plasma exposure has a significant inhibitory effect on T98G cell growth and may have genotoxic effects on the cell. The obtained results of the growth kinetics, MTT and clonogenic assays are correlated to each other. The 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. Clonogenic assay is the method to determine cell reproductive (mitotic) death after treatment. After plasma treatment, only a fraction of the seeded cells retained the capacity to produce colonies among living cells. So finally, we can conclude that plasma was not only inducing death in T98G cells, but also significantly affecting the reproductive capacity of the cells and has a broad exposure dosage ranges of activity against the human T98G brain cancer cells. This inhibitory effect on the clongenicity of gliomas also indicates genenetic damage or modification induced by plasma. Further studies on genetic damage or genotoxcity by plasma on gliomas is under progress.

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