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Generation of Atmospheric Pressure Dry- and Mist-Plasma Jets and Their Effects on HeLa Cells

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ABSTRACT: Atmospheric pressure plasma jets have recently received significant attention due to their unique capabilities and novel applications. Various chemical species, such as NO_2, HNO_3, O_3, and OH, generated in air by plasma are dissolved and transported in water quickly, and accompanied chemical stimuli can inactivate bacteria. In our previous study, focusing on OH and H_2O_2 production, we developed a mist-plasma jet (MPJ) generated using dry helium gas mixed with pure water mist as an alternative to the traditional method using only dry helium gas, known as the dry-plasma jet (DPJ). In this study, the observation and comparison of effects of both MPJ and DPJ on HeLa cells surrounded by cell culture medium immediately after irradiation by plasma and following 24 h were focused. In addition, to observe details of effects of both plasma jets on HeLa cells, two experimental procedures were prepared. One is that of irradiated culture medium, including that with cells and observed cells replaced with fresh cell culture medium following 24 h. The other is that of irradiated culture medium, without cells, with plasma, and observed cells exposed to the plasma-treated culture medium after 24 h. These experiments revealed that MPJ more greatly influences cell death than DPJ.

KEY WORDS: dry-plasma jet, mist-plasma jet, HeLa cells, cell death ratio

I. INTRODUCTION

Atmospheric pressure plasma is nonthermal, high pressure, and is capable of producing UV radiation, charged particles, and reactive species, which is a high-velocity effluent stream.[1,2] The plasma can be touched by bare hands without any feeling of electrical shock or warmth.[3] Because of these characteristics, the atmospheric pressure plasma jets have great potential in various fields for applications of biology and medicine such uses as sterilization,[4,5] disinfection,[6–8] and tooth whitening.[9,10] Furthermore, cancer therapy[11–13] and wound healing[13–17] have been expected using the plasma jet and actively studied. Because it is well known that atmospheric pressure plasma is effective to inactivate microorganisms, the idea arose to try to use the plasma on sensitive living surfaces or wounds for antisepsis. Therefore, the main focus of plasma medical applications is to treat infectious skin diseases or wound healing. Among these studies, a classification related to the quality of contact of the plasma with the target has been discussed. Fridman et al.[18] suggested that the plasma treatments were distinguished in two types, i.e., direct

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or indirect. Direct treatment means biological samples or living tissue serve as one of the electrodes necessary for plasma ignition. In indirect plasma treatment, the electrodes are part of the plasma-generating device, only. Thus, there is primarily no electrical contact to the target.[18] In addition, important for fields of sterilization or cell activation, according to Hamaguchi,[19] many types of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are specifically produced in water by plasma jet irradiation, with these reactive species playing an important part in sterilization or cell activation. The hydroxyl radical (OH) in particular plays an important role in plasma chemistry and plasma medicine due to an oxidation potential and disinfection power substantially higher than other oxidative species.[20] In our previous study, the mist-plasma jet (MPJ), generated using dry helium gas mixed with pure water mist, as an alternative to the traditional method using only dry helium gas, known as the dry-plasma jet (DPJ), was developed to enhance efficiency of OH production amount.[21] This study focuses on the observation and comparison of the effects of both MPJ and DPJ on HeLa cells surrounded by cell culture medium immediately after irradiation by plasma and following 24 h.

II. METHODOLOGY

A. Cell Cultivation

This study utilized the HeLa cell, which is a cell type in immortal human cell line and used commonly in biochemical and medical science.[22–24] The cells were cultivated in a cell culture dish (Φ35) (2-8590-01, AS ONE Corp., Japan) containing 2 ml (1.5×10^5 cells/ml) with a regular medium that consists of Dulbecco's modified Eagle medium (D-MEM 043-30085, Wako Pure Chemical Industries, Ltd., Japan), 10% fetal bovine serum (FBS, SH30910.03, HyClone Laboratories, Inc., United States), and 1% penicillin-streptomycin (168-23191, Wako Pure Chemical Industries Ltd., Japan) for 24 h in an incubator (WKN-9100EX, Waken B Tech Co., Ltd., Japan) at 37°C with 5% CO_2.

B. DPJ and MPJ Generation System

Figure 1 is a schematic diagram of the experimental setup of DPJ and MPJ generation. DPJ was generated using only dry helium gas by directly connecting a dry helium gas cylinder, a mass flow controller (Model 8500, KOFLOC Co., Ltd., Japan), and an alumina tube. Conversely, MPJ was generated using helium gas to which pure water mist was added by installing an atomizing device between the mass flow controller and the alumina tube. The plasma jet system consists of a high-frequency, high-voltage power supply (PHF-2K, Haiden Kenkyuusyo Co., Ltd., Japan), an alumina tube, a high-voltage electrode, and a ground electrode placed on the tube at a certain distance of separation. For stable generation of the plasma jet, an aluminum plate, which connects to ground, with a 10 mm thickness, was set under the plasma jet system. The aluminum plate was set on an acrylic plate of 10 mm. The applied voltage and operating frequency were fixed at 11.5 kV and 1.0 kHz, respectively. The target was placed on the aluminum plate

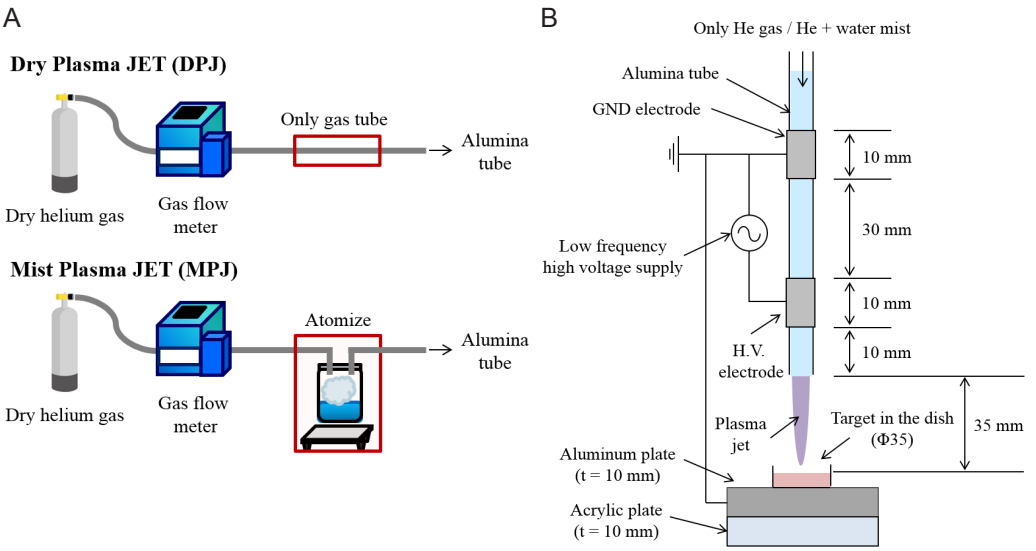


FIG. 1: Schematic diagram of the experimental setup of DPJ and MPJ generation. (A) Definition of DPJ and MPJ; (B) plasma jet system.

and irradiated by the plasma jet for 5 min because some reported that effects of plasma treatment with 5 min against biofilms[25] and chronic wounds[26] were confirmed; in addition, it freed of side effects to wound healing.[27]

C. Measurement of Cell Death Ratio

To confirm cell death ratio, nuclei of prepared cells were stained by Acridine Orange/Propidium Iodide (AO/PI) (F23001, Logos Biosystems, Inc., Annandale, VA, USA). AO is permeable to both live and dead cells and able to stain nuclei to generate green fluorescence. PI enters dead cells with compromised membranes and is able to stain nuclei of dead cells to generate red fluorescence. After the staining, the cells were moved to a cell counting slide (L12001, Logos Biosystems, Inc.) and its cell viability was measured by a LUNA-FL Dual Fluorescence Cell Counter (L20001, Logos Biosystems, Inc.), which captures and analyzes three different images, bright field, green fluorescence, and red fluorescence, and subsequently these cell images are analyzed with accurate image analyzing software to calculate the cell viability. In this study, cell death ratio was calculated as 100% minus the cell viability. To reduce the variability among the samples, three samples were used for each treatment and the mean number of each group that was treated with DPJ was compared with that of the samples treated with MPJ by Student's *t*-tests (two-sided unpaired sample), where the level of significance was set at 0.05. The error bar for each replication was determined from standard deviation (SD).

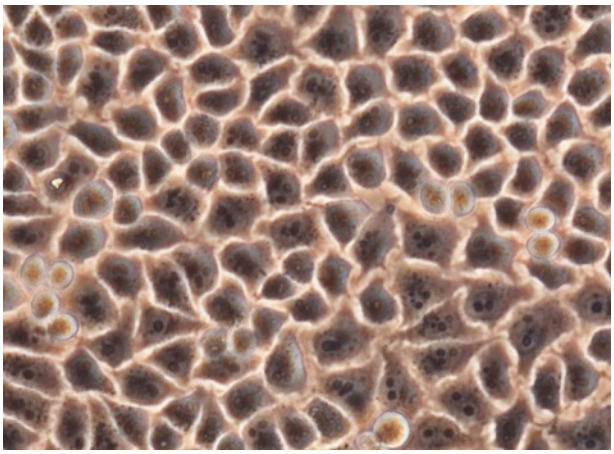


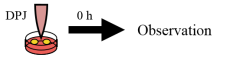
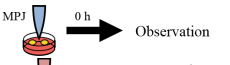
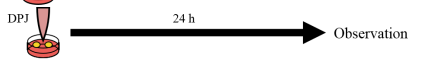
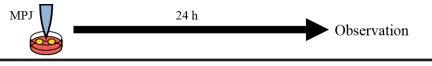
FIG. 2: Representative morphology of HeLa cells used for plasma irradiation experiments

III. RESULTS AND DISCUSSION

A. Cell Death Ratio Immediately and 24 h after the Plasma Jets Treatment

To investigate the effects of DPJ and MPJ to the HeLa cells, the objects were analyzed via the fluorescence cell counter immediately and 24 h after the treatment. Figure 2 shows the representative morphology of HeLa cells used for plasma irradiation experiments. The prepared cells were divided into six groups, which are shown in Table 1. Group Nos. 1 and 2 are control and positive control, the cells exposed to 35% H_2O_2 (Kishida Chemical Co., Ltd., Osaka, Japan) and observed immediately after the irradiation, respectively. Group Nos. 3 and 4 are observed immediately after DPJ and MPJ treatment, respectively. Group Nos. 5 and 6 are observed 24 h after DPJ and MPJ treatment, respectively. Figure 3 shows the results of cell death ratio in each group. The cell

TABLE 1: Experimental groups of cell death ratio immediately and 24 h after the irradiation

No.	Affected part by PJ	Experimental procedure
1	–	Control
2	–	Positive control
3	Cells and culture medium	
4	Cells and culture medium	
5	Cells and culture medium	
6	Cells and culture medium	

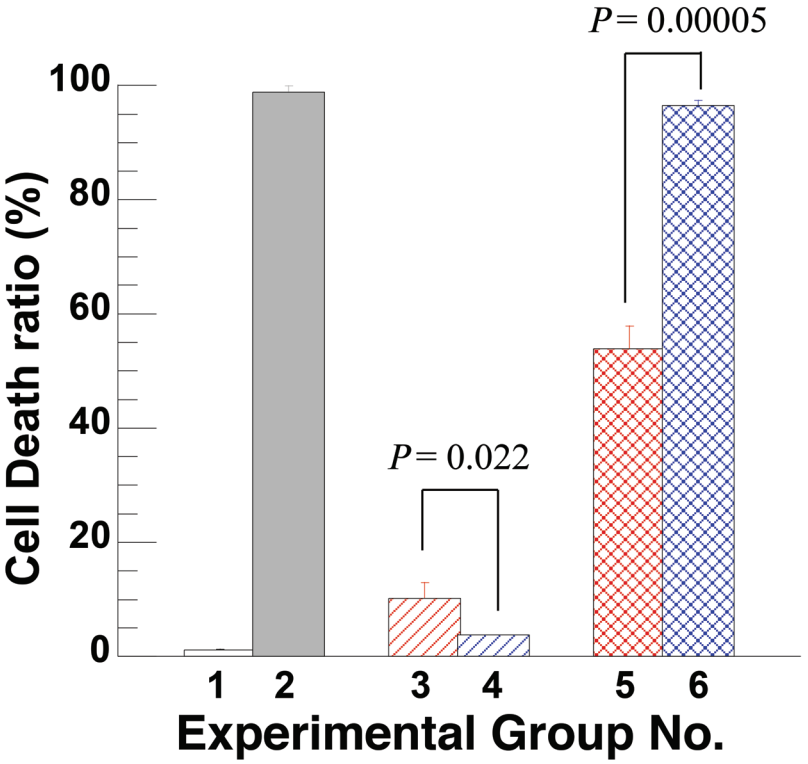


FIG. 3: Cell death ratio of the experimental groups Nos. 1–6

death ratio increased to 1.1, 98.8, 10.2, 3.8, 53.9, and 96.5% as a result of group No. 1 to No. 6, respectively.

According to Nos. 1 and 2, it was confirmed that adequateness of prepared cells and staining procedure were proved for this study.

According to Nos. 3 and 4, they indicated little effect on cell death immediately after both treatments. In addition, DPJ treatment had significance compared to MPJ treatment. These results suggested that direct stress caused by plasma and chemical effects in the culture medium during the irradiation had little effect on the cell death. Furthermore, DPJ is more effective than MPJ treatment even in low effects on cell death.

According to Nos. 5 and 6, they indicated significant effect on cell death 24 h after both treatments. Furthermore, MPJ had significance compared to DPJ treatment. These results suggested that direct stress and chemical effects in the culture medium until the observation had great effects on the cell death, especially MPJ treatment.

B. Temperature of Cultural Medium Irradiated by Plasma Jets

To examine the case of cell death immediately after both treatments, temperature of

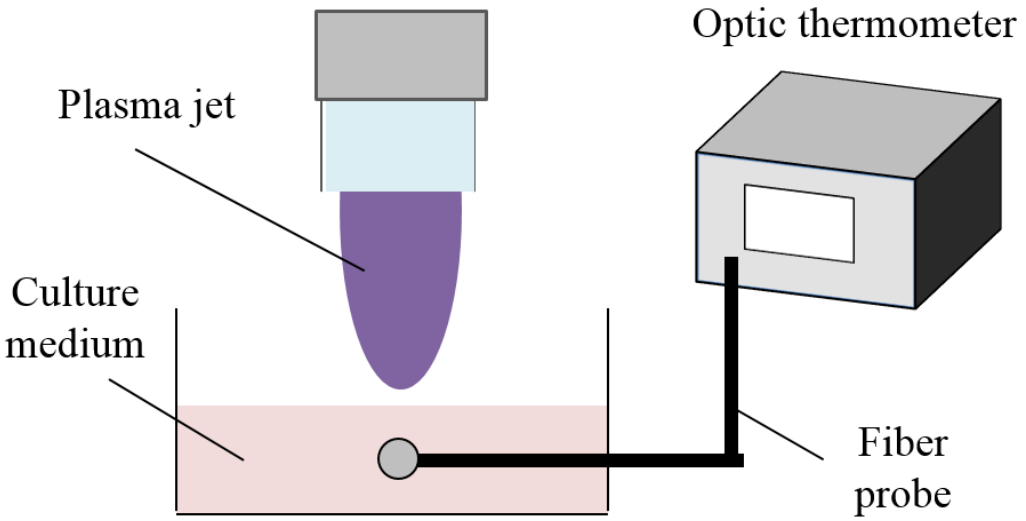


FIG. 4: Experimental setup for measuring the temperature of the culture medium

the cultural medium was measured by an optical thermometer (FL-2000, Anritsu Meter Co., Ltd., Tokyo, Japan) during the irradiation. Figure 4 shows the setup for the measurement. A fiber probe (FSE-35380, Anritsu Meter Co., Ltd.), connected to the optic thermometer, was placed in the center of the cell medium through a hole on the side of the dish. The measurement was carried out until the temperature was saturated.

Figure 5 shows the result of temperature of the culture medium during both irradiations. The temperature of the culture medium irradiated by DPJ was increased gradually. On the other hand, the temperature of the culture medium irradiated by MPJ was invariable or prevented. The temperature in the case of DPJ irradiation after 3 min reached 61.5°C. This result indicated that one of the factors of significance of cell death ratio immediately after DPJ treatment compared with MPJ was the increased temperature of culture medium induced by DPJ. In addition, due to mixed pure water mist, it was obviously confirmed that MPJ irradiation to the culture medium had invariable temperature or was able to prevent the increase in temperature of culture medium.

C. Effects of the Primary and Secondary Reactions on Cell Death Rate

To obtain a deeper insight into the influence of both treatments to the cell death of Nos. 5 and 6, four groups were prepared (Table 2). In this study, the authors defined direct stress caused by plasma and chemical effects in the culture medium during the irradiation as the "primary reactions," and also defined chemical species dissolved by plasma jet in the culture medium as the "secondary reactions." For examination of the primary reactions, culture medium including cells was irradiated with DPJ (No. 7) and MPJ (No. 8). Irradiated cells were replaced with fresh culture medium and observation was car-

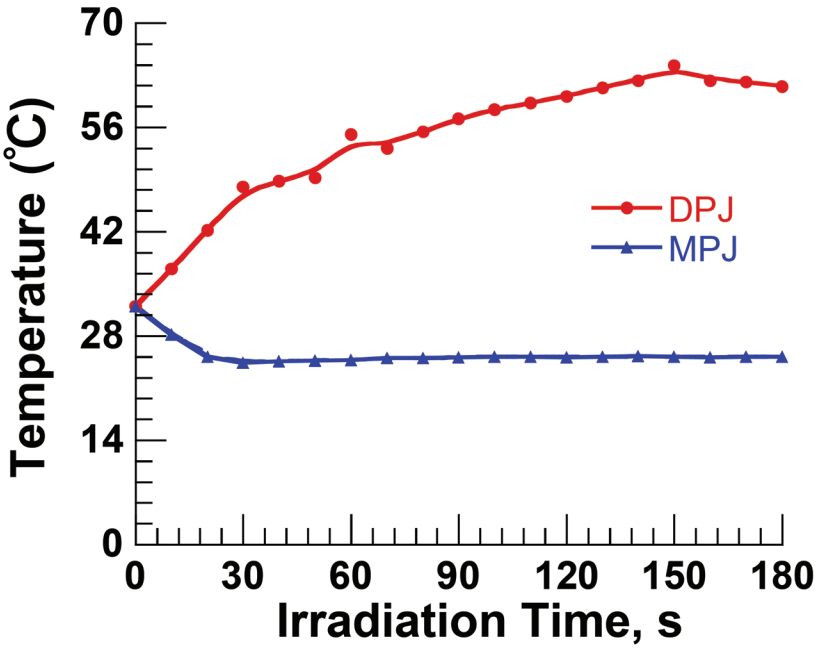


FIG. 5: Temperature of the culture medium during the irradiation

ried out after 24 h. For examination of the secondary reactions, cultural medium without cells irradiated with DPJ (No. 9) and MPJ (No. 10) were used. The cells were cultivated in plasma-treated culture medium and both cells were observed after 24 h.

Figure 6 shows the result of cell death ratio. The cell death ratio increased to 8.6, 2.6, 26.3, and 89.9% as a result of group Nos. 7–10, respectively. Thus, this results suggested that the secondary reactions of plasma to cell death is much higher than the primary reactions. Although there were little primary effects of plasma to cell death,

TABLE 2: Experimental groups of cell death ratio for distinguishing primary and secondary reactions

No.	Affected part by PJ	Experimental procedure
7	Cells	DPJ → Replaced with fresh culture medium → 24 h → Observation
8	Cells	MPJ → Replaced with fresh culture medium → 24 h → Observation
9	Culture medium	DPJ → 24 h → Observation
10	Culture medium	MPJ → 24 h → Observation

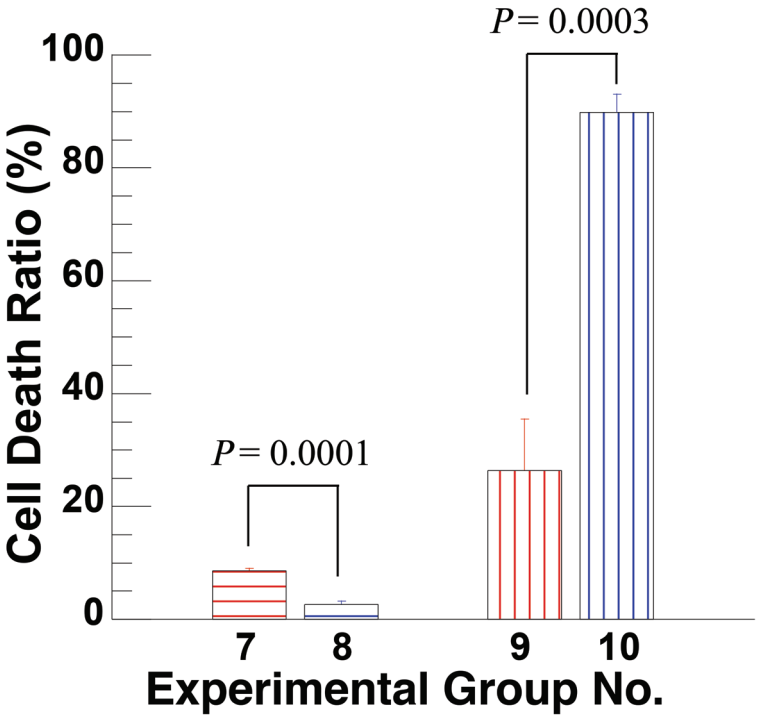


FIG. 6: Cell death ratio of the experimental groups Nos. 7–10

DPJ treatment (No. 7) has significance compared with MPJ (No. 8). As already mentioned, this significance can be explained as induced by the increased temperature of culture medium treated with DPJ. In secondary reactions, it has significant effects on cell death and MPJ has significant effects compared with DPJ according to Nos. 9 and 10, respectively. These results are suggested to have been induced due to chemical species produced by plasma. Discharge in gaseous phase produces chemical species such as $^1O_2$ (singlet oxygen), O_3 (ozone), OH (hydroxyl radical), O_2^- (superoxide), HO_2 (hydroperoxy radical), H_2O_2 (hydrogen peroxide), NO (nitrogen monoxide), and NO_2 (nitrogen dioxide).[19] According to Kanazawa et al., the main reactions relating to OH radicals in the pulsed discharge are[28]

$$e + H_2O \rightarrow H + OH + e \quad (1)$$

$$O(^1D) + H_2O \rightarrow 2OH \quad (2)$$

$$N_2(A^3\Sigma^+) + H_2O \rightarrow N_2 + OH + H \quad (3)$$

$$OH + OH \rightarrow H_2O_2 + (M) \quad (4)$$

$$OH + OH \rightarrow H_2O + O \quad (5)$$

$$OH + H + (M) \rightarrow H_2O + (M) \quad (6)$$

$$OH + H \rightarrow H_2O \quad (7)$$

$$OH + O \rightarrow O_2 + H \quad (8)$$

where $O(^1D)$, $N_2(A^3\Sigma^+)$, and M indicate excited state of the oxygen atom, metastable state of nitrogen atom, and any inert molecule, respectively. In our previous study, it was confirmed that DPJ and MPJ can generate OH radicals.[21] Shimizu et al.[29] reported that the concentrations of H_2O_2, O_3, and HNO_2 in the treated water increased as the discharge time. In addition, Yonemori et al.[30] reported that when helium is artificially humidified using a water bubbler, the OH density increases with humidity. Furthermore, some reported that morphological damage processes in both cases of plasma-treated and H_2O_2-added culture media showed the same trend of the decrease in the cell survival ratio correspondingly[31] and H_2O_2 is one of the main factors responsible for inactivation of HeLa cell viability.[32] Therefore, it was predicted that the chemical species that dissolved in the culture medium were one of the main factors responsible for cell death according to Nos. 9 and 10. Moreover, due to mixed water mist, OH radicals were more generated by MPJ that might be caused OH radical reactions and H_2O_2 might be increased in the culture medium. For this reason, MPJ treatment (No. 10) had significant effects on cell death compared with DPJ (No. 9).

IV. CONCLUSION

This study focuses on the observation and comparison of effects of both MPJ and DPJ on HeLa cells surrounded by cell culture medium immediately after irradiation by plasma and following 24 h. The results suggested that significant effects on cell death were observed 24 h after both treatments, while little effects on cell death were observed immediately after the irradiation. To examine the case of significance of DPJ on the observation immediately after the treatment, the temperature of culture medium was measured by the optic thermometer during the irradiation until the temperature was saturated. The results indicated the temperature increased in DPJ treatment, moreover, it was obviously confirmed that MPJ irradiation to the culture medium had invariable temperature or was able to prevent the increase in temperature of culture medium. From these results, one of the factors responsible for cell death observed immediately after the treatment is due to increased temperature of the culture medium irradiated by DPJ. To obtain a deeper insight into the influence of both treatments to the cell death observed 24 h after the treatment, in addition, the authors defined direct stress caused by plasma and chemical effects in the culture medium during the irradiation as the primary reaction, and also, defined chemical effects dissolved by plasma jet in the culture medium. For distinguishing the primary and the secondary reactions, four groups were prepared and compared (shown Table 2). The results indicated that the secondary reactions of plasma to cell death is much higher than the primary reactions; furthermore, MPJ treatment had significance compared to DPJ treatment. Thus, it was suggested the main cause of cell death is secondary reactions, and MPJ is more effective to kill HeLa cells due to mixed pure water mist. Qualification of OH production in DPJ and MPJ as well as OH and H_2O_2 in culture medium after DPJ and MPJ irradiation is required as future work.

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