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# Characterization of Cold Atmospheric Pressure Plasma Technology and Its Anticancer Properties

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**ABSTRACT:** The anticancer properties of plasma were studied by treating Dulbecco's modified Eagle's medium (DMEM) with cold atmospheric pressure plasma (CAPP). The CAPP was generated by using high voltage power supply (11.75 kV) at an operating frequency of 50 Hz. The DMEM was treated with cold plasma using argon as the process gas for the different exposure time ranging from 0.5 to 3 minutes. The treated media were transferred to Henrietta Lacks (HeLa) and Human Embryonic Kidneys 293 (HEK 293) cells. The viability of cancer cells was observed using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cold atmospheric pressure plasma discharge has been characterized by electrical and optical methods. The cold plasma treatment selectively killed cancer cells without affecting normal cells *in vitro*. It has been observed that the percentage viability of the cell lines varies with the plasma treatment time along the best fitted curve of a power function. The curve is steeper for the cancer cells than for the normal cells after plasma treatment. The faster decaying curve signifies the selective killing of the cancer cells compared to the normal cells within the exposure time. This study indicates that the reactive oxygen species in the CAPP activate the apoptosis pathway in the cancer cells. As a novel strategy, using the CAPP stimulated media has become a promising anti-cancer tool.

**KEY WORDS:** cold atmospheric pressure plasma, anticancer properties, MTT assay, HeLa cells, HEK 293, DMEM

## I. INTRODUCTION

A plasma reactor applicable for the medical purpose can be designed with locally available materials. The plasma device is able to generate the non-equilibrium atmospheric pressure argon plasma of low temperature (24–27°C) downstream using a high voltage power supply (11.75 kV) operating at a frequency of 50 kHz. A cost-effective system of generating cold plasma at atmospheric pressure has potential applications in biomedical research.[1,2] Cold atmospheric pressure plasma (CAPP) is a partially ionized gas comprising ions, electrons, UV-photons and reactive neutrals such as radicals, excited and ground state molecules. It is gaining more attention these days because of its widespread applications such as surface modification, food

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preservation, microbial decontamination and cancer treatment.[3] In case of CAPP, the plasma discharge is faster and the electrons and heavy particles are in thermal non-equilibrium leading to much lower temperature of the heavy particles than that of the electrons.[4,5] CAPP consists of many reactive species including oxygen or nitrogen based radicals.[6] These components of CAPP and their complicated chemistry lead to a myriad of interaction with biological systems including cells and tissues.[6–8] CAPP with argon gas as its working environment has the ability to cause cell detachment and successfully kills the cancer cells. The principles of CAPP have been used in multiple medical fields and have become a promising medical technology.[9,10] Cold argon plasma generating devices are safe and easy to operate and can now be manufactured at a low cost due to advancements in electronics and microchips. CAPP can combat tumor growth by increasing the efficacy of antitumor therapeutic agents, reactivating apoptotic pathways, or down regulating growth related gene sites.[11] Cancer is abnormal growth of cells which is usually derived from a single abnormal cell having genetic defects. The cells have lost the normal control mechanism and thus are able to multiply continuously, invade nearby tissues, migrate to distant parts of the body, and promote the growth of new blood vessels from which the cells derive nutrients. Cancerous cells can develop from any tissue within the body. As cancerous cells grow and multiply, they form a mass of cancerous tissue called a tumor that invades and destroys normal adjacent tissues.[12,13] According to the data of WHO, cancer is the second leading cause of death, and it has become the most prevalent disease of this modern world. It is very difficult to diagnose, treat and cure cancer. Many researches are going on for the better diagnosis, treatment and cure of cancer. One of the promising therapeutic ways is use of CAPP treatment which is relatively young so that it may need additional research.[14,15]

## II. MATERIALS AND METHODS

### A. Plasma Treatment Setup

A typical experimental setup used for treating Dulbecco's modified Eagle media (DMEM) is as shown in Fig. 1. The entire reactor system is fixed on a movable table. The reactor consists of a transparent polycarbonate cylinder of height 10 cm, diameter 10 cm and thickness 2 cm. The edges of the cylinder are smoothed using the lathe machine. Both the upper and lower electrodes are made up of brass (5.1 × 5. 1 × 1 cm). A polycarbonate sheet of 2.2 mm thickness is inserted between the two electrodes which serves the purpose of the dielectric barrier. The dielectric sheet helps to limit the current and helps in making the discharge uniform. The electrodes are connected to 10 kΩ resistor which is then connected to a high voltage transformer. The reactor system has two pipes fitted. One of the pipes is connected to a vacuum pump while the other pipe is connected to the analogue pressure gauge. For treating DMEM, argon was used as a process gas at 11.75 kV keeping distance between media and electrode 1.5 cm.

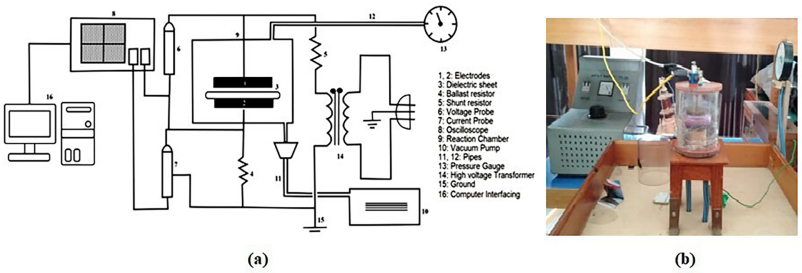



**FIG. 1:** (a) Schematic diagram of the experimental setup and (b) image of the CAPP discharge in working condition (plasma activated media for DMEM treatment)

## III. METHODS

### A. MTT Assay

A colorimetric assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used for the quantification of viability of the cell lines. Here, MTT was reduced to formazan by living cells. Formazan is an insoluble crystalline product with deep purple color. Basically, viable cells contain an enzyme like nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) dependent oxidoreductase which is responsible for reducing MTT into formazan. Formazan was dissolved by dimethyl sulfoxide (DMSO). Then, absorbance of the dissolved formazan was taken at 500–600 nm using a plate reader. Larger number of living cells in wells means the greatly reducing MTT into the formazan product with darker solution.[16,17]

### B. DMEM Treatment

The HeLa cells and HEK293 cells were seeded in 96 well plate at the density of 10,000 cells per well. 10% DMEM media kept 2.5 ml at each petri plates were treated with plasma produced from parallel plate DBD for 0.5, 1, 1.5, 2, and 3 minutes, respectively. Original normal media was removed from seeded plates and 100 µL plasma treated media was transferred to each well of 96 wells plate. 100 µL of untreated media was added to control cell lines. The viability of each cancer cell lines was quantified. MTT assay was performed after incubating plasma treated cell lines for 24 hours under standard conditions. The percentage of survived cells after treatment was determined from the average absorbance taken at 570 nm of each test and then it was compared with control cells.[17]

## IV. RESULTS AND DISCUSSION

### A. Electrical Characterization

The value of electron density ($n_e$) has been calculated by using Power Balance Method.[18]

This method is based upon the principle that the energy lost by the plasma parameters is equal to the power delivered by the source. The balance between the input power from high voltage power supply and power lost in the plasma is expressed as same equation expressed in Eq. (1).

$$n_e = \frac{P_{av}}{2Av_bE_{lost}} \tag{1}$$

Figure 2 shows the current-voltage waveforms of DBD at frequency 50 Hz and working voltage 11.75 kV. The calculation of electron density was made by using the values given below. The electrodes distance was 3 mm apart, applied frequency was provided 50 Hz, cross section area of each electrode = 20.43 $cm^2$, Bohm velocity is to be taken ($v_b$) = $2 \times 10^3$ m/sec[19](almost constant in case of argon discharge), energy lost per cycle is $E_{lost}$ = 50 eV. Using these values in Eq. 1, the electron density ($n_e$) was found to be $2.52 \times 10^{13}$ $cm^{-3}$ at atmospheric condition.

Discharge power is measured by the integration of instantaneous voltage $V(t)$ and current $I(t)$.[20]

$$\text{Discharge power, } P(w) = f\int_0^T V(t)I(t)\,dt \tag{2}$$

where $f$ is the frequency and $T$ is the time of the cycle. We used the trapezoidal rule for the calculation with $V(t)$ and $I(t)$; the data recorded through oscilloscope. Using values of $V_{rms}$ = 11.75 kV and $I_{rms}$ = 2.5 mA in Eq. 2, the power consumed per cycle was found to be 29.37 watt.

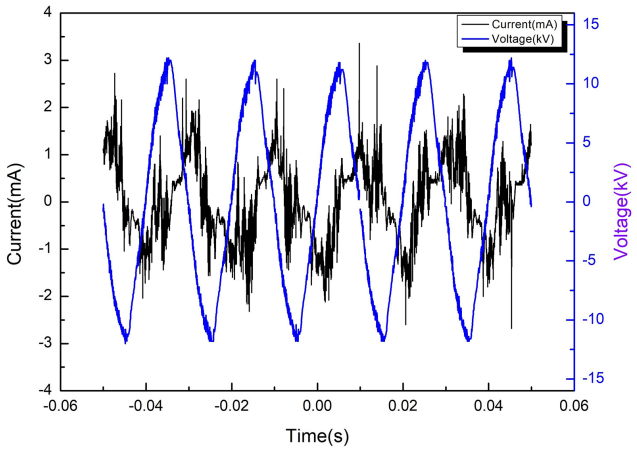



**FIG. 2:** Current-voltage waveforms of DBD at atmospheric condition at 11.75 kV (50 Hz)

## B. Optical Characterization

For the optical characterization of dielectric barrier discharge (DBD) plasma, a small hole is made on the cylindrical polycarbonate tube and an optical fiber is inserted there. The discharge of the DBD is made to pass through the optical fiber and the spectra are recorded by the Ocean Optics (USB2000+) at atmospheric conditions. Figure 3 shows the spectra of discharge as a plot for the intensity wavelength at an atmospheric pressure condition. The flow rate of argon was 3 L/min. Four suitable argon lines, two for Ar I (696.47 nm and 750.705 nm) and two for Ar II (314.8 nm and 379.81 nm), are chosen from the spectra and the electron temperature $T_e$ is estimated using the line intensity ratio method given by Eq. 3.[21,22]

$$\frac{R_1}{R_2}=\frac{I_1/I_2}{I_3/I_4}=\left(\frac{A_{pq}}{A_{xy}}\right)\left(\frac{g_p}{g_x}\right)\left(\frac{\lambda_{xy}}{\lambda_{pq}}\right)\left(\frac{A_{uv}}{A_{rs}}\right)\left(\frac{g_u}{g_r}\right)\left(\frac{\lambda_{rs}}{\lambda_{uv}}\right)exp\left[-\frac{E_p-E_x-E_r+E_u}{K_BT_e}\right] \quad (3)$$

where $R$ is the ratio of intensity of two lines, $I$ is the intensity of the spectral line, $A_{ji}$ is the transition probability of the transition $i \rightarrow j$, gi is the statistical weight of the upper level, λ is the wavelength of the line radiation, $E_i$ is the energy of the upper level, $K_B$ is Boltzmann constant and $T_e$ is the electron temperature. The values of λ and $I$ are obtained from the observation, and the corresponding values of the transition probability, statistical weight and energy levels for the argon I and II lines were obtained through (NIST) Atomic Spectra Database.[23] During argon discharge at atmospheric condition, electron temperature was estimated, assuming that the local thermodynamic equilibrium (LTE) was obtained.

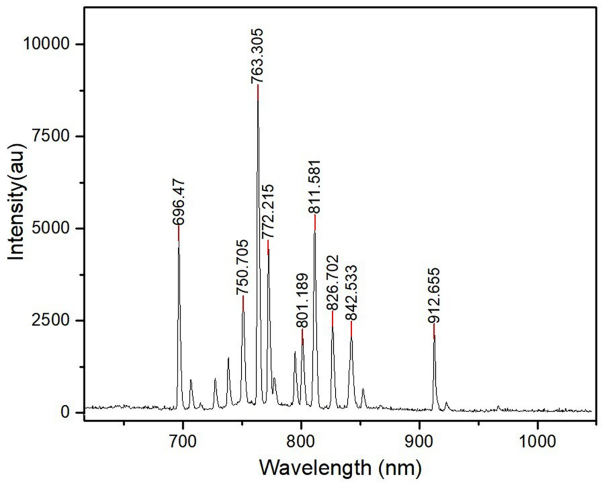



**FIG. 3:** Spectra of the discharge of frequency 50 Hz at an applied voltage of 11.75 kV in argon environment [gas flow rate (Q) = 3 L/min]

Figure 4 shows the plot of intensity ratio $R_1/R_2$ vs. electron temperature $T_e$. The value of $T_e$ has been found to be 0.98 eV, which corresponds to the low temperature plasma applicable for the treatment of cancer (HeLa) and normal (HEK 293) cells.

## C. Anticancer Efficacy of CAPP

The microscopic view of cancer cells (HeLa) and normal cells (HEK293) after 24 hours incubation with plasma treated media for different exposure time to CAPP is shown in Fig. 5a and 5b. Figure 6a and 6b, shows the coloration developed after putting formazan in MTT treated normal and cancer cells. From the bar diagram (Fig. 7), there is no significant difference in viability of cancer and normal cell line when treated for 0.5 minute. As the time of treatment increases from 0.5 to 3 minutes, there is significant difference in viability between cancer cells and normal cells.

It has been observed that there is selective inhibitory effect on both HeLa and HEK293 cells with increase in treatment time. The HEK293 cells are less sensitive

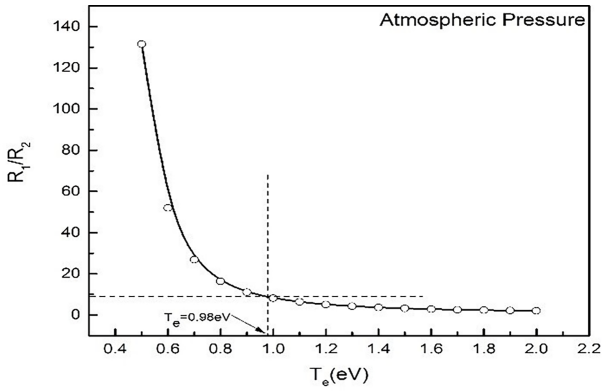
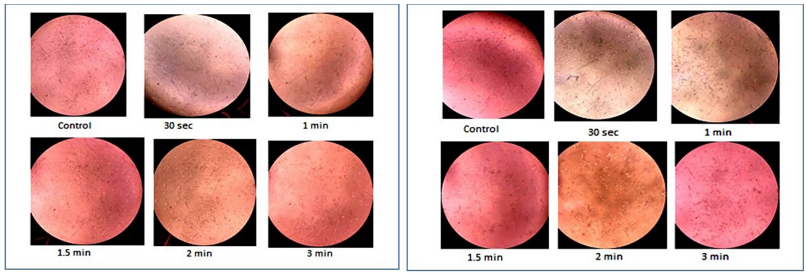
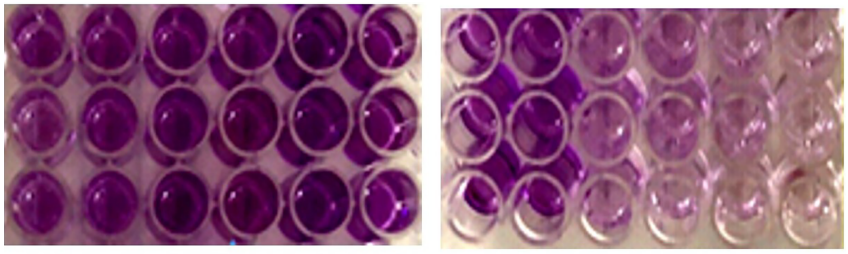



**FIG. 4:** Plot of intensity ratio $R_1/R_2$ as a function of electron temperature $T_e$




(a) (b)

**FIG. 5:** Microscopic view of normal cells (HEK293) (a) and cancer cells (HeLa) (b) after 24 hours of incubation with plasma-treated medium for different exposure times



(a) (b)

**FIG. 6:** Coloration after adding formazan in MTT-treated normal cells (HEK293) (a) and cancer cells (HeLa) (b)

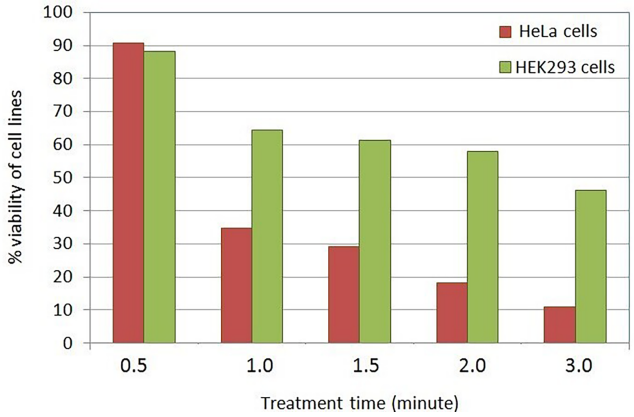



**FIG. 7:** Comparison of percentage viability of cancer cells (HeLa) and normal cells (HEK293) after transferring plasma-treated media through bar diagrams

to the CAPP treated media than HeLa cells at all treatment time periods. The anti-proliferative effect in the cells depends on the time period of DMEM media treatment by CAPP. When the media treatment time reaches 3 minutes, 89.1% of HeLA cells become dead, however in case of HEK293 cells only 53.7% of them are found to be dead.

The selective effect of CAPP seen on normal and cancer cells is due to the significant differences in the biochemical structures.[24] Cancer cells and normal cells differ in the phases of cell cycle as a higher percentage of cancer cells are in the S phase of cell cycle rendering them more susceptible to the CAPP treatment.[25] There is a cell cycle checkpoint between S and G2 phase. It signifies that each phase of the cell cycle is accurately completed before progression into the next phase. The CAPP treatment causes DNA damage in the cancer cells leading them to apoptosis. The hydrogen peroxide produced in CAPP gets faster diffusion in the cancer cells than in normal cells due to the presence of reactive species in the cancer cells.[6]

While treating the cells with CAPP, the experimentally observed data points of % viability of cell lines ($y$) versus exposure time ($x$) are best fitted with the power function described by Eq. 4.

$$y = Ax^{-P} \tag{4}$$

where $A$ is a constant and $P$ is the power raised to the exposure time ($x$). For the plasma treatment of normal cells (HEK293), A = 68.87 ± 1.6 and P = 0.33 ± 0.03. this way, for the plasma treatment of cancer cells (HeLa), A = 39.74 ± 2.2 and P = 1.17 ± 0.08. where the included errors are the standard errors in the related coefficients of the power functions. Here, the coefficient of determination is $R^2 = 0.96$ for HEK 293 and for HeLa. From the interpretation of Eq. 4 and as reflected by Fig. 8, the percent viability of normal cell lines decreases gently with the exposure time whereas the % viability of cancer cell lines decreases steeply with the exposure time to CAPP. Thus, we can say that CAPP selectively kills cancer cells (HeLa) compared to normal cells (HEK293). The CAPP technology with argon gas as a working environment has the ability to deactivate microorganisms, cause cell detachment, cause death in cancer cells and can combat tumor growth by increasing the efficacy of antitumor therapeutic agents.[26–28]

## V. CONCLUSION

CAPP is produced and characterized by electrical and optical methods. Electron density ($n_e$) and electron temperature ($T_e$) of cold plasma have been observed to be $2.52 \times 10^{13}$ $cm^{-3}$ and 0.98 eV in plane parallel DBD by using power balance and intensity ratio methods respectively. The experiments clearly show the strong anticancer efficacy of

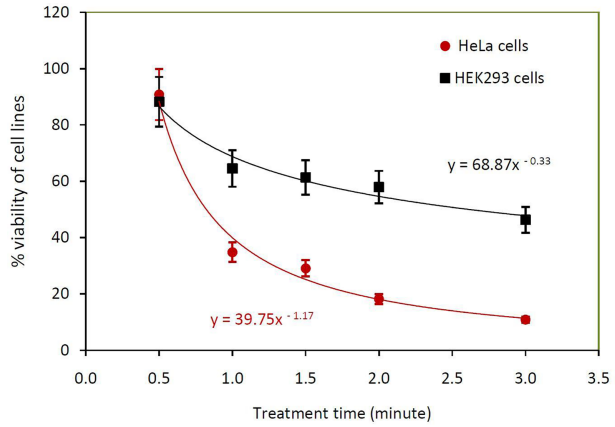



**FIG. 8:** Comparison of percentage viability of cancer cells (HeLa) and normal cells (HEK293) after transferring plasma-treated media through the best fitted power functions shown with error bars

CAPP. CAPP can be the best plausible method of surface sterilization. While treating normal (HEK 293) and cancer (HeLa) cells with CAPP *in vitro*, the percentage viability of the cell lines follows the nature of a power function with respect to the treatment time. The curve of such power function is steeper for the cancer cells (HeLa) than for the normal cells (HEK293). The results suggest the significant difference in viability between cancer cells and normal cells as the exposure time increases from 0.5 to 3 minutes. It offers the selective killing of cancer cells compared to normal cells. In conclusion, the CAPP treated media can be a promising alternative tool for curing various types of internal cancers with minimal damage to the surrounding cells.

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