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Antimicrobial and Anticancer Efficacy of Atmospheric Pressure Cold Plasma Technology

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Abstract: The present work evaluated the inactivation efficiency of atmospheric pressure cold plasma (APCP) against three pathogenic microorganisms. We have successfully developed a plasma device that can generate atmospheric pressure cold argon plasma of low temperature (24^0 - 27^0C) downstream using a high-voltage power source (6 kV) operating at a frequency of 19.56 kHz, which can be widely used in biomedicine. Therefore, a cost-effective system of generating cold plasma jets at atmospheric pressure with potential applications in biomedical research has been developed. The discharge has been characterized by an optical method. This research aims to investigate the antimicrobial property shown by the atmospheric pressure cold plasma Jet (APCPJ). Three pathogenic bacterial strains (*Shigella flexneri, Escherichia coli, and Klebsiella pneumoniae*) were used to test the antimicrobial property. The effects of atmospheric pressure cold plasma (APCP) today have been identified worldwide in disinfection, decontamination, and sterilization, as well as oncology applications. This work aims to demonstrate the effect of APCP irradiated media as a promising anticancer tool. Atmospheric pressure cold plasma technology has the efficacy to show anticancer properties with the treatment of cancer cells. To demonstrate the anticancer properties of APCP generated at a high voltage power supply (12 kV) at an operating frequency of 50 Hz. 10% Dulbecco's Modified Eagle Medium (DMEM) media were treated with cold plasma using argon as a process gas for various time durations (0.5-4 min). The treated media was transferred to Henrietta Lacks (HeLa) and Human Embryonic Kidneys 293(HEK 293) cells, and the viability of cancer cells was observed using MTT assay.

Keywords: atmospheric pressure cold plasma; MTT assay; HeLa cells; HEK 293; DMEM media; Antimicrobial and anticancer efficacy of cold plasma; *Shigella Flexneri*; *Escherichia coli; Klebsiella pneumoniae.*

1. Introduction

Atmospheric pressure cold plasma jets (APCPJs) are capable of generating non-thermal plasma needles that are not confined by electrodes, which gives them a lot of potential for biomedical applications. In the present work, the antimicrobial efficiency of APCPJ was tested against three pathogenic microorganisms with different cell wall properties [1]. Most of the previous study has used RF power supply which is more expensive than the power supply used in the present study. The plasma jet is constructed with locally available materials and can be

made treatment continuous for a long time. We have successfully developed a plasma device that can generate a non-equilibrium atmospheric pressure argon plasma jet of low temperature, which can be widely used in biomedical applications [2]. Therefore, a cost-effective system of generating cold plasma at atmospheric pressure with potential applications in material processing and biomedical research has been developed [2,3]. Atmospheric pressure cold plasma (APCP) is a partially ionized gas comprising ions, electrons, ultraviolet photons, and reactive neutrals such as radicals, excited, and ground-state molecules. These days, it is gaining more attention because of its widespread application, such as surface modification, food preservation, microbial decontamination, and cancer treatment [4]. Bacteria such as *Shigella flexneri, Escherichia coli, and Klebsiella pneumoniae* may also play a role in chronic wound infection. The widespread application of systemic and topical antibiotics has led to the emergence of resistant bacterial strains [4,5]. APCPJ can be used as an alternative to a chemical wound disinfectant. We aim to study the antibacterial effect of APCPJ generated using argon as process gas against three different bacterial strains: *Escherichia coli, Shigella flexneri, and Klebsiella pneumoniae* [6,7].

In the case of cold atmospheric plasma, the atmospheric pressure plasma discharge is fast. The electrons and heavy particles are in thermal non-equilibrium, leading to a much lower temperature of the heavy particles than that of the electrons. The heavy particle temperature of APCP varies between 24°C and 45°C, which makes it easier to be used in biomedicine [8]. APCP consists of many reactive species, including oxygen-based radicals, nitrogen-based radicals, and other components [9,10]. These components of APCP and their complicated chemistry lead to a myriad of interactions with biological systems, including cells and tissues [10,11]. In this research, we used argon gas as a working environment. APCP can deactivate microorganisms, cause cell detachment, and cause death in cancer cells.

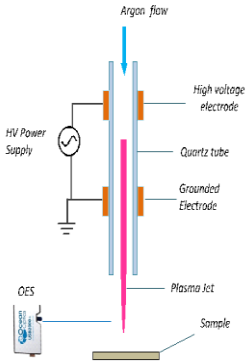
Atmospheric pressure cold plasmas (APCP) have been used in multiple medical fields and have become a promising medical technology [12]. Cold argon plasma generating devices are safe and easy to operate and can now be manufactured at a low cost due to advancements in electronics and microchips. APCP can combat tumor growth by increasing the efficacy of antitumor therapeutic agents, reactivating apoptotic pathways, or down-regulating growth-related gene sites [13]. The abnormal growth of cells derived from the single abnormal cell is known as a cancer cell. The cells have lost their normal control and can multiply regularly, spread out nearby tissues, and move to the far distant parts of the body. Finally, cancer cells grow and multiply to form a mass of cancerous tissue called a tumor that invades and destroys normal adjacent tissues [14,15]. According to the data of WHO, cancer is the second leading cause of death and the most disease of the modern world. It is very difficult to diagnose, treat and cure cancer. Research are going on for the better diagnosis, treatment, and cure of cancer. One of the promising therapeutic ways is cold atmospheric pressure plasma treatment; the field is relatively young and needs further research [15-17].

2. Materials and Methods

2.1. Materials and methods to study the antimicrobial properties of APCPJ.

The schematic diagram of the experimental setup and the nature of the discharge is shown in figure 1(A&B). The experimental setup consists of a capacitive coupled inner electrode system made of quartz tubes of outer diameter 4 mm and inner diameter 3 mm. The copper electrode is connected to a high voltage power supply (0-20 kV).

The distance between the two electrodes is fixed at 3.5 cm, and the distance between the nozzle of the plasma jet from the lower electrode is 0.3 cm. The process gas during the experiment is argon, maintained its flow rate is 5 L/min, and working voltage and operating frequency are maintained at 6 kV and 19.56 kHz, respectively. The optical characterization of the discharge has been done using an optical emission spectrometer (USB 2000+).



(A)

(B)

Figure 1. (A) Schematic diagram of the experimental setup and (B) image of the discharge [2].

2.2. Materials and methods to study the anticancer properties of APCP.

Figure 2 shows the schematic diagram of plane parallel DBD, consisting of (1,2) Electrodes (3) Dielectric Sheet(4) Ballast Resistor (5) Shunt Resistor (6) Voltage Probe (7) Current Probe (8)Oscilloscope (9) Reaction Chamber (10) Vacuum Pump (11,12) Pipes (13) Pressure Gauge (14) High Voltage Transformer (15) Ground (16) Computer.

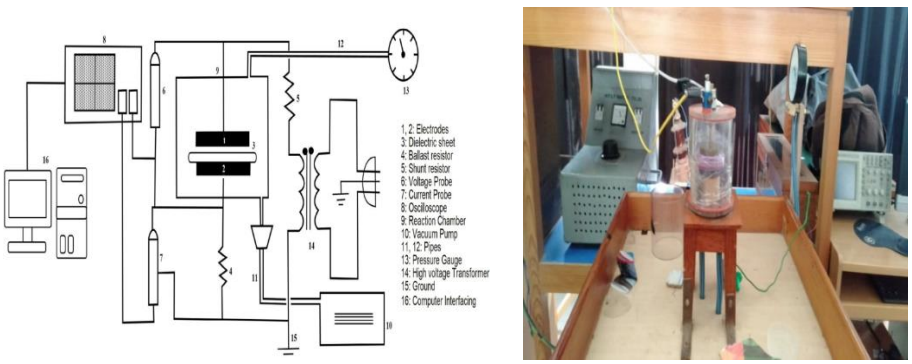


Figure 2. Schematic diagram of the experimental setup and image of the discharge.

The typical experimental setup used for treating DMEM media is as shown in figure 2. The entire reactor system is fixed on a movable table. The reactor consists of a transparent polycarbonate cylinder of height 9.5 cm, diameter 9.5 cm, and 1.8 cm thickness. The edges of

the cylinder are smoothed using the lathe machine. Both the upper and lower electrodes are made up of brass (5.1×5.1×1cm). A polycarbonate sheet of 2.2 mm thickness is inserted between the two electrodes, which serve 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 a 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 analog pressure gauge. For treating DMEM media, argon was used as a plasma source at 12 kV, keeping the distance between media and electrode 1.5 cm.

3. Results and Discussion

3.1. Optical characterization of the discharge (Jet).

During optical characterization of the plasma discharge, in oder to calculate electron temperature, the line intensity ratio method was used. In this method, two Ar I and two Ar II lines were chosen from the obtained spectra. The working formula for the calculation of the electron temperature is expressed as [18,19].

$$\frac{R_1}{R_2} = \frac{I_1/I_2}{I_3/I_4} = \left(\frac{A_{pq}}{A_{xy}}\right)\left(\frac{g_p}{g_x}\right)\left(\frac{\lambda_{xy}}{\lambda_{pq}}\right)\left(\frac{A_{uv}}{A_{rs}}\right)\left(\frac{g_u}{g_r}\right)\left(\frac{\lambda_{rs}}{\lambda_{uv}}\right) exp\left[-\frac{E_p - E_x - E_r + E_u}{K_B T_e}\right] \quad (1)$$

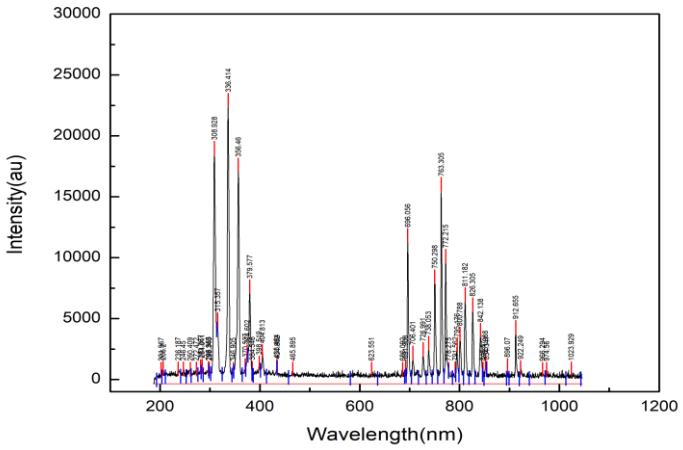


Figure 3. Spectra of the discharge at frequency 19.56 kHz and an applied voltage of 6 kV in the Argonenvironment(flow rate =5 L/min).

Figure 3 shows the discharge spectra at frequency 19.56 kHz and an applied voltage of 6 kV at an argon flow rate of 5 L/min.

Table 1. Intensity and wavelength of ArI and ArII lines.

Lines	Intensity(au)	Wavelength(nm)	Ratio
ArI	11685.20	696.54	I_1
ArI	8317.36	749.87	I_2
ArII	4820.17	314.82	I_3
ArII	7407.65	379.81	I_4

Table 1 shows the intensities of argon lines at corresponding wavelengths. Substituting values of different parameters obtained from NIST atomic database in equation (1), we have

$$\frac{R_1}{R_2} = 1.14\times10^{-2} \exp\left[\frac{2.524}{kT_e}\right] \quad (2)$$

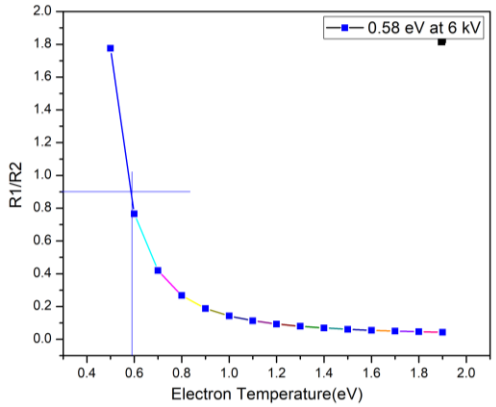


Figure 4. The plot of R1/R2 as a function of electron temperature.

For, $\frac{I1/I2}{I3/I4}$ = 0.91, from figure 4, electron temperature is to be measured at about 0.58 eV.

3.2. Temperature measurement of the atmospheric pressure cold plasma jet.

Atmospheric pressure plasma jet approximately matches the surrounding atmosphere. This plasma is also called (cold) normal plasma. The plasma jet was designed with locally available materials to make treatment cost-effective and continuous. The temperature of the plasma jet was directly measured, which was about 27^0 C at discharge voltage 6 kV, being called atmospheric pressure cold plasma jet and widely used in bio-medical fields [2,20]. Figure 5 shows the variation of plasma jet temperature with time at discharge voltage 6 kV. It was linear for up to 60 seconds, then after almost remained constant at 27^0 C from 90-180 seconds.

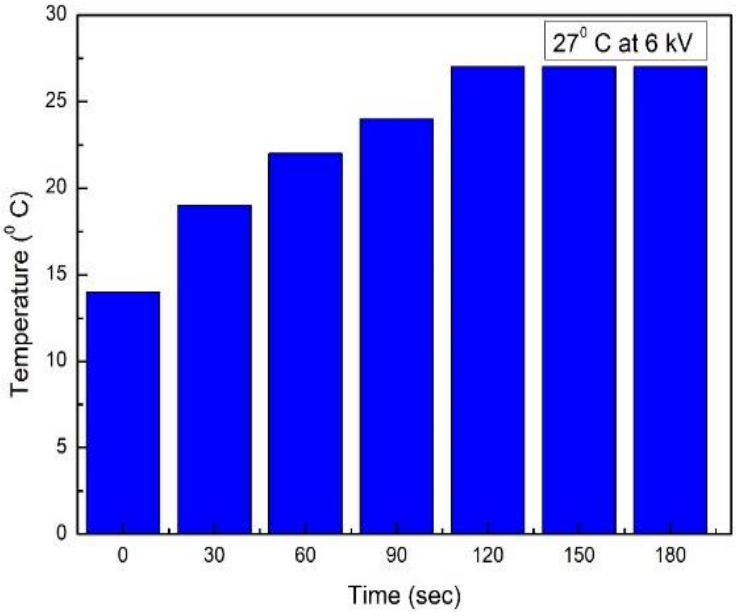


Figure 5. Graph of the temperature of the plasma jet as a function of time at 6 kV.

3.3. Antimicrobial efficacy of APCPJ.

Figure 6(A&B) shows the discharge image during treatment and antimicrobial activity of the cold plasma jet against *E. coli*, *S. flexneri*, and *K. pneumonia* for the control and treated samples at 6 kV. Bacterial Strains were obtained from ATCC Culture Bank. Then, it was grown in Muller Hilton Agar Medium in agar plates and kept for one-hour incubation in the

refrigerator at 4^0 C. These samples were treated by APCPJ after one hour. These treated plates were incubated for 24 hours at 37^0 in an incubator. Finally, the zone of inhibition (ZOI) formed in these treated samples was measured. APCPJ showed the best ZOI 13.6±1.1 mm against *K pneumoniae* when treated for 20 sec at 6 kV. The generated cold argon plasma had an antibacterial effect that was directly dependent on applied voltage and treatment time.

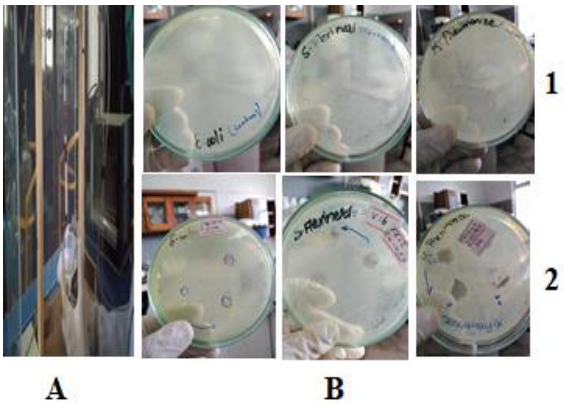


Figure 6. (A) Image of the discharge and (B) Antimicrobial activity of the cold plasma jet against *E. coli*, *S. flexneri* and *K. pneumoniae*. 1 is control, and 2 shows the zone of inhibition for 10 sec, 15 sec, and 20 sec in an anticlockwise direction.

Table 2. Data of zone of inhibition with treatment time at 6 kV.

Treatment time(sec)	Zone of inhibition in mm (*E. coli*)	Zone of inhibition in mm (*S. flexneri*)	Zone of inhibition in mm (*K. pneumoniae*)
10	5.3	9.6	10.6
15	6.6	11.1	13.4
20	7.2	12.9	13.8

Table 2 shows the measurement of the zone of inhibition (ZOI) formed by treatment using the cold argon plasma jet for 10, 15, and 30 seconds with a burner distance of 3cm.

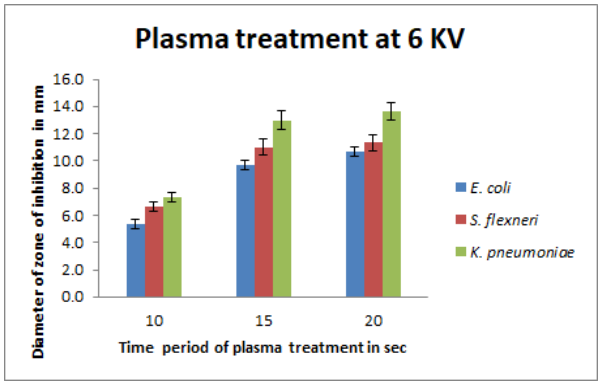


Figure 7. The diameter of zone of inhibition of different bacteria strains with treatment time at 6 kV.

Figure 7 shows the antimicrobial efficacy of the atmospheric pressure cold plasma jet (APCPJ) at 6 kV with treatment times 10 seconds, 15 seconds, and 20 seconds. Moreover, APCPJ can be the best plausible method of surface sterilization. The most commonly used disinfectant and topical antibiotics can negatively affect tissue health and tissue recovery. APCP is made up of atoms, positive and negative ions, free radicals, excited and non-excited molecules, and even photons. The significant attribute of cold argon plasma jet is that it doesn't have any negative effects compared to other tropical disinfectants. As we increased treatment time, more electrons, positive ions, and reactive oxygen species get accumulated in a petri dish containing strains of pathogenic bacteria which in turn showed a larger zone of inhibition of

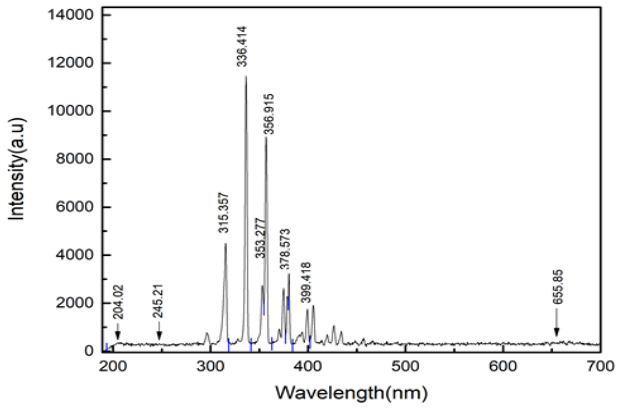
bacterial growth and from the above data that *E. coli* is gaining more resistant against antibacterial medicine [21].

3.4. Optical characterization of DBD.

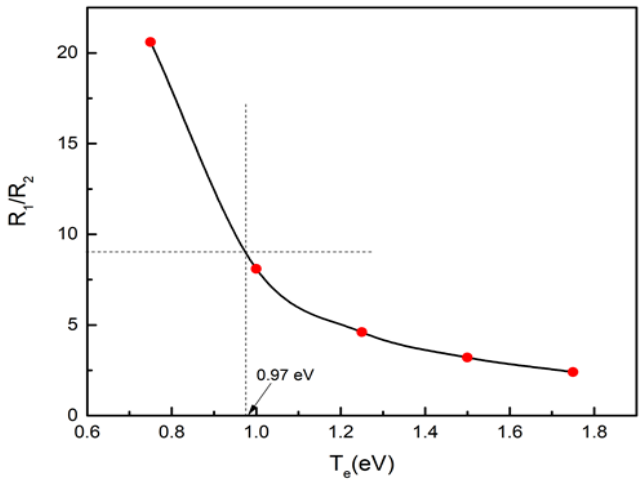
For optical characterization of DBD, a small hole is made on the cylindrical polycarbonate tube and an optical fiber is inserted there. The discharge of the DBD is made to pass through the optical fiber, and the spectra are recorded by the Ocean Optics (USB2000+) at atmospheric conditions. Figure 8(A) shows the spectra of the discharge and their corresponding intensities and wavelength at an atmospheric pressure condition. In this method, four suitable lines were chosen from spectral lines obtained from the discharge. The optical characterization is carried out using the line intensity ratio method expressed in equation (1) [22].

In the above equation (1), R be the ratio of the intensity, I be the intensity of spectral line, A_{ji} being the transition probability from $i \rightarrow j$, g_i be the statistical weight of the upper level, λ being the wavelength of the radiation, E_i being the energy of the upper level, K_B and T_e are the Boltzmann constant and electron temperature respectively. The values of λ and I are taken from the observation, and the values of A_{ji}, g_i and E_i were taken from NIST atomic spectra database [23].

(A)



(A)



(B)

Figure 8. (A) Spectra of the discharge at 50 Hz and 12 kV and (B) Graph of the ratio of intensities with electron temperature.

From figure 8(B), it is seen that the electron temperature for atmospheric pressure cold plasma was found to be 0.985 eV.

3.5. Anticancer properties of atmospheric pressure cold plasma (APCP).

MTT assay is a widely used assay for quantifying the viability of cell lines. This method of the viability of cells quantification was first described by Tim Mossman in 1983. In this colorimetric assay, yellow tetrazolium salt (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, or MTT) is reduced to formazon by living cells. Formazon is an insoluble crystalline product with deep purple color. Formazon is dissolved by dissolving solvents like isopropyl alcohol or DMSO. Then absorbance of the dissolved formazon is taken at 500- 600 nm using a plate reader. The more the living cells in wells, the greater the MTT dye up taken by living cells and converted into formazon [24].

3.5.1. Cell culture.

The cells received at a particular passage number were subjected to several step-by-step methods to grow and harvest cells optimally. Such a method includes cell revival, cell trypsinization, maintenance, and cryopreservation.

3.5.2. Cell revival.

Six milliliters of 10% DMEM were taken and incubated for 30 minutes at 37 degrees Celsius and 5% CO_2 in a CO_2 incubator. The cryopreserved cells were thawed in a water bath at 37°C. The thawed cell suspension was transferred into medium and centrifuged at 1500 rpm, 25° C for 7 minutes. In T 25 cell culture flask, 1mL of suspension was mixed with 5 mL of 10% DMEM and kept for incubation in a CO_2 incubator. Respective media change was done on the next day. Cells were passaged at the sub-confluent stage.

3.5.3. Cryopreservation.

The cells were harvested by centrifuging at 1500 rpm, 25° C, for 7 minutes. The cell pellet was re-suspended in 500 µL of 10% DMEM. An equal volume of the freezing mix (90% FBS + 10% DMSO) was added to the cryovials and mixed properly. The cryovials were transferred to the styrofoam box, kept at -20°C for 2 hours, and transferred to -80°C overnight. Afterward, the vials were cryopreserved in a liquid nitrogen tank for long-term storage and use.

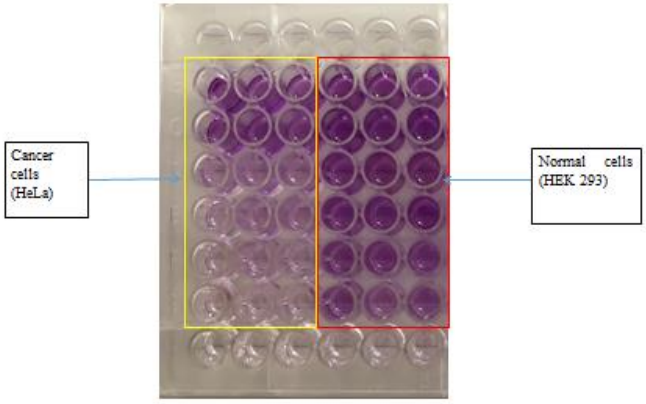


Figure 9. Coloration developed after putting formazon in MTT treated cancer cells(left) and normal cells(right).

3.6. Cold plasma technology treatment of cell lines.

Figure 9 shows the coloration developed after putting formazon in MTT treated normal and cancer cells. HeLa cells and HEK 293 cells were seeded in 96 well plates at the density of 10,000 cells per well. 10% DMEM media kept 2.5 ml at each Petri plate were treated with a plasma produced from parallel plate DBD for 0.5 minutes, 1 minute, 1.5 minutes, 2 minutes, 3 minutes, and 4 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 line was quantified. MTT assay was done then after incubating plasma-treated cell lines for 24 hours under standard conditions. After treatment, the percentage of survived cells was determined from the average absorbance taken at 570 nm of each test and was then compared with control cells [24].

Table 3. Percentage viability of cell lines after transferring plasma-treated media.

Time of Treatment	**% viability of cells**	
	HeLa Cells	HEK293 cells
control	100	100
0.5 min	91.2	88.2
1 min	35	64.8
1.5 min	29	61.4
2 min	18.2	58.1
3 min	11.2	46.1
4 min	10.9	45.9

Table 3 shows the variation of % viability of cancer cells (HeLa) and normal cells (HEK 293) as a function of treatment time.

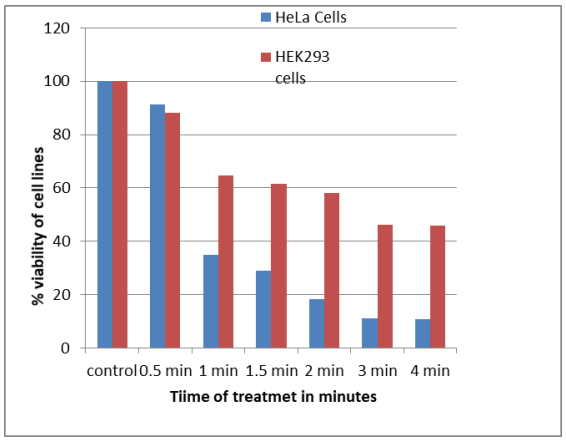


Figure 10. Comparison of cell percentage viability of cancer cells and normal cells after transferring plasma-treated media.

Figure 10 shows that 10% of DMEM media was treated with atmospheric pressure cold argon plasma at 12 kV for a time duration of 0.5 minutes to 4 minutes. The treated media was transferred to HeLa and HEK 293 cells seeded in 96 well plates. After 22 hours of incubation, the MTT assay was performed to see the viability of cells lines. As shown in table 3 and the bar graph in figure 10, there was no significant difference in the viability of cancer and normal cell line when treated for 0.5 minutes. As the time of treatment increases from 0.5 minutes to 4 minutes, there is a significant difference in viability between cancer cells and normal cells. So, we can say that atmospheric pressure cold plasma was selectively killing cancer (HeLa) cells compared to normal cells (HEK 293), which is clearly shown in figure 9. In this

experiment, we used argon gas as a working environment. APCP technology can deactivate microorganisms, causes cell detachment, causes death in cancer cells, and can combat tumor growth by increasing the efficacy of antitumor therapeutic agents [25,26]

4. Conclusions

Most of the previous work has used an RF power supply that is more expensive than the power supply used in the present study. Therefore, a cost-effective system of generating plasma technology at atmospheric pressure with potential applications in biomedical research has been developed. The discharge temperature was measured to be about 270C, so the produced discharge is termed cold plasma. Atmospheric pressure cold argon plasma has been produced and characterized by an optical method. Electron temperature (T_e) was found to be of the order of 0.58 eV (APCPJ) and 0.985 eV (plane parallel DBD), respectively, using the intensity ratio method. The results from the experiment clearly showed the strong antibacterial properties of APCPJ. It was found that the antimicrobial efficacy increases with the increase in voltage and time of exposure. The effect of cold plasma jet treatment, *E. coli* was found most resistant, and *K. pneumoniae* was found most sensitive to the treatment. The effect of plasma treatment for *S. flexnari* lay in between them. Moreover, the used APCP can be the best plausible surface sterilization method. The result suggests the significant difference in viability between cancer cells and normal cells as the time of treatment increases from 30 seconds to 4 minutes, and the selective killing of cancer (HeLa) cells compared to normal cells (HEK 293) was observed. The results from the experiment clearly showed the anticancer properties of atmospheric pressure cold plasma.

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Conflicts of Interest

The authors declare no conflict of interest.

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