Effects of a Nonthermal Atmospheric Pressure Plasma Discharged in Cell Culture Medium on Melanoma Cell

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Abstract: Cold atmosphere plasma has been developed and applied as a novel technology for cancer treatment. In most researches cap was directly applied to irradiate cancer cells or tissues. In this study, we report that CAP plasma discharge in cell culture medium and studied its effects on melanoma cancer cells. CAP plasma treated cell culture medium was applied to melanoma and fibroblast cells. Cell viability test by using MTT assay showed that treatment of with CAP plasma had the strongest effect on inducing apoptosis to inhibit melanoma cell proliferation and further cell metastasis. The most important is that CAP plasma treatment do not have any harmful effect on normal fibroblast cell, but do have exhibited strong cell killing effect on B16/F10 melanoma cell.

Key words: Cold atmospheric plasma, non-invasive treatment, melanoma cell.

1. Introduction

Cancer is a disease of unlimited cell division while the normal cell has the ability of self-regulation allowing for appropriate and controlled cell division and growth. Current treatment strategy for cancer is based on surgery to remove cancer cells. In order to eradicate residual cancer cells to reduce the risk of recurrence, an adjuvant therapy after curative surgery is used [1]-[4]. However, adjuvant treatments such as chemotherapeutic drugs have negative side effects as they also kill normal cells due to their high proliferation. Therefore, the development of a novel therapeutic method for killing target cells is strongly needed. As a novel therapeutic technique, cold atmospheric pressure plasma jets has been introduced as a new tool with potential for cancer treatment [5]-[8] due to their many advantages, which include operation at atmospheric pressure, application to target sites without high heat damage [9]-[12].

In most researches, CAP plasma was directly applied on cells or tissues. Direct application of CAP plasma limits its therapeutic ability on the deeper part of cancer cells or tissues [13]. In this study, we report that CAP plasma discharge in cell culture medium and studied its effects on cancer cells. A home-made CAP device was designed to generate plasma. The CAP stimulated cell culture medium can be applied to cells and tissues when direct access by plasma is not available. This paper describes the plasma device, its characterization, and the response of cancer cells following CAP plasma treated cell culture medium application.

2. Experimental Design

Submit your manuscript electronically for review. The cold atmosphere plasma jet device was made in lab
and it consists of contains a centrally powered electrode with a ground outer electrode wrapped around a quartz tube (9 mm in diameter) with a jet nozzle (3.4 mm in diameter). The electrodes were connected to a high voltage power supply, helium gas (He) was used as carrier gas and the volume flow rate was controlled by using mass flow rate controller. The flow rate of helium gas was set between 3 to 5 slpm and the working distance between CAP jet and cell culture medium was 2 cm. A thermocouple temperature sensor was used to measure the temperatures after CAP plasma has activated with distance of 20 mm from the CAP jet nozzle.

Optical emission spectroscopy (OES) was used to assess various species in CAP plasma. Here, OES results was shown as qualitative analysis but not quantitative results. In the present work, a wavelength of 200 to 1000 nm was investigated to detect various atmospheric plasma species including atomic oxygen [O], hydroxyl radical [OH\(^-\)], nitrogen [N\(_2\)] and helium [He]. Optical emission spectroscopy (OES) was comprised of a collimating lens with diameter of 15 mm, an optical fiber probe and an optical emission spectrometer (Ocean optics QE65000). The optical probe was placed 2 cm in front of the plasma jet nozzle.

The B16/F10 melanoma cell was purchased from the Culture Collection and Research Center (Hsinchu, Taiwan). The B16/F10 cells, 4 x 10\(^5\) cells, are seeded at 100 mm culture dishes containing 7 ml of DMEM with 10% fetal bovine serum (FBS). Cells are then cultured at 37°C in a humid atmosphere containing 5% CO\(_2\). Cells are passed twice a week and the culture medium was changed every two days for cell maintenance. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay (Sigma-Aldrich M2128), a colorimetric assay for detecting the activity of cell mitochondria, was used to evaluate cell viability. Cells are seeded into 24-well plate in the density of 10\(^4\) cells/well with 1 ml DMEM contains 10% FBS then cultured in a 37°C incubator. Cells were then incubated for one day to ensure proper cell adherence and stability. Then we put plasma treated culture medium solutions into the wells to check their effects on the cells. After additional incubation at 37°C, the original culture medium was aspirated, and the MTT solution was added to each well. After another 4 hrs, the medium was replaced by MTT solvent ((0.4% (v/v)) HCl in anhydrous isopropanol) to dissolve formazan crystals. Then, solution absorbance was determined by using ELISA reader at 570 nm.

The CAP plasma treatment time effect on cell viability was performed. We first seed 10\(^4\) cells/well in 24-well plate with 1 ml DMEM contains 10% FBS and culture for 24 h in the 37°C incubator. Cells are treated with CAP plasma for 60, 120, 180, 240, 300 and 420 sec respectively then cultured for 24 h in the 37°C incubator. Finally, the cell viability is determined by MTT assay.

The cell migration assay was also performed to evaluate the effect of CAP treated cell culture medium on melanoma cells. Cells are seeded into 24-well plate in the density of 10\(^4\) cells/well with 1 ml DMEM contains 10% FBS then cultured in a 37°C incubator for 24 h. Then one artificial wound per well is scratched into the monolayers with a sterile plastic 1000 μl micropipette tip to generate a uniform wound. After a wash with PBS two times and refill with 0.6 ml culture medium, we use CAP plasma treat for 180 and 360 sec respectively. The result will record at 0, 6, 12, 24 and 48 h and quantified by Image J software.

3. Results and Discussions

The optical characteristics of CAP plasma discharged are shown in Fig. 1. The identification of emission lines of reactive species was analyzed based on [14]. From the emission spectrum, OH peaks can be found at 297 and 309 nm, lines in the range of 600 to 800 nm are He gas, peaks at 316, 337, and 358 nm are the result of the formation of N\(_2\) second-positive system. Overall higher emission intensity was found with higher helium gas flow rate.

In order to investigate the physical and chemical properties change of cell culture medium after CAP plasma treatment, we measure the temperature and pH value of cell culture medium during CAP plasma
treatment, as shown in Fig. 2. After 30 to 360 sec CAP plasma treatment, the temperature of the culture medium is still maintained between 24 to 30 °C which is lower than body temperature 37°C and will not cause thermal damage to living cells even death. The pH value of cell culture medium after CAP plasma treatment is kept between 8.4 to 8.5. As compared with the control group, the variation of pH value with different plasma treatment time is only ±0.6% and will not have any negative effect on cell viability.

![Emission spectrum of CAP plasma with a helium gas flow rate of 3, 4 and 5 slpm.](image1)

**Fig. 1.** Emission spectrum of CAP plasma with a helium gas flow rate of 3, 4 and 5 slpm.

![Temperature and pH value of cell culture medium after CAP plasma treatment with treatment time from 60 to 360 sec.](image2)

**Fig. 2.** Temperature and pH value of cell culture medium after CAP plasma treatment with treatment time from 60 to 360 sec.

We further investigate the effect of CAP plasma treatment time, cell culture medium volume and seeding cell number on melanoma cell by MTT assay. The results were shown in Fig. 3. As the CAP plasma treatment time increases, the melanoma cell viability decreases to 30%. As the treatment time increases to 180 sec, the cell viability decreases to 52%. So that the median lethal dose was set to be 180 sec of plasma treatment time. The cell culture medium volume treated with CAP plasma has a strong effect on the cell viability. The cell viability increases as cell culture medium volume increase until the medium volume is 0.6 mL. As medium volume is larger than 0.6 mL, the cell viability is kept almost at the same value. That means the minimum volume of medium for stable cell culture is 0.6 mL. The communication and interaction between cells are affected by cell density that results in the change of cell behavior and also the viability. The results show that at lower cell seeding number, 10000, the cell viability decreases dramatically with the increasing CAP plasma treatment time. But at higher cell seeding number, 50000, the cell viability seems not be
affected with the increasing CAP plasma treatment time. The reason is that at higher cell density, CAP plasma cause cell damage may reduce immediately by cell-cell interaction [15]. Thus, CAP plasma treatment time of 180 and 360 sec, cell culture medium volume of 0.6 ml and cell seeding number of $10^4$ were selected as the experimental condition of CAP plasma for further experiments.

Fig. 3. Effect of (a) CAP plasma treatment time (b) cell culture medium volume and (c) cell number on the viability of melanoma cell.

Tumor metastasis occurs with a series of steps such as cell migration, adhesion, proliferation, invasion, and vessel formation through a complex mechanism including microenvironment interaction, gene expression, and enzymes regulation. We investigated the CAP plasma treatment effects on the suppression
of melanoma cell migration behavior via a migration assay. The results are shown in Fig. 4. In the results, melanoma cells migration ability is significantly suppressed in both 180 and 360 sec CAP plasma treatment groups and the quantified result is also shown in Fig. 4. The cell uncovered area ratio is used as an index for representing the cell migration ability. That means the lower uncovered area ratio the higher cell migration ability. After culture of 24 hrs, the cell migration ability of 180 sec CAP plasma treatment group decreased by 1.4 folds meanwhile 360 sec treatment group reduced by 2.3 folds compared with the control group. These results indicated that CAP plasma treatment can suppress the migration of melanoma cells. When integrin expression decrease will cause cell adhesion increase and migration decrease, conversely, integrin expression increase will cause cell adhesion decrease and migration increase [15]. So that CAP plasma treatment can alter cell surface receptor function to lower integrin expression and further suppress cell metastasis.

![Cell Migration Ability Test](image)

Fig. 4. Effect of CAP plasma treatment on the migration ability of melanoma cells. The cell uncovered surface area ratio was chosen as an index for representation of cell migration ability.

In order to verify that CAP plasma treatment has no negative effect on normal tissues, the cell viability test of L929 fibroblast cell was conducted. Fig 5 shows the results of effect CAP plasma treatment on the viability of melanoma cell and fibroblast cell. The results show that viability of melanoma cell can be strongly affected by the treatment of CAP plasma. With the treatment time of 180 and 360 sec, the cell viability is down to 60% after 3 day culture. But the cell viability of fibroblast cell is not affected by the treatment of CAP plasma. The results show that CAP plasma treatment do not have harmful effect on normal cells/tissues, but do have obviously suppressed effect on cancer cell proliferation.

It has report that cancer therapy, such as immunotherapy, chemotherapy and irradiation, exert their anti-cancer effect by induction of apoptotic cell death [16], [17]. Apoptosis is promoted by signaling stimulus through specific cell surface receptors then trigger a cascade of intracellular molecules and initiate the cell death program [18], [19]. When cells undergoing apoptotic process usually involve initial disruption of cell-cell contacts, nuclear condensation, nuclear fragmentation, and packaging of cellular organelles into membrane-bound vesicles. As a next step to examine the apoptotic cell death, we perform by Hoechst 33258 staining which a fluorescent stain for labeling DNA and visualize nuclei. Data shows that, in the control group, cells are remaining in typical round nuclei indicate that the cells are in a healthy state (Fig.6). Contrarily, both 180 sec and 360 sec CAP plasma treatment group, cells have appeared nuclear
condensation which represent of apoptosis. We also observed that cell number are significant reduce. It may due to cell death which suspend and remove with culture medium. Thus, it's indicate that CAP plasma treatment can induce melanoma cell apoptosis.

Fig. 5. Effect CAP plasma treatment on the viability of (a) melanoma cell and (b) fibroblast cell.

4. Conclusion

In summary, this study investigated the effect of CAP plasma discharged in cell culture medium on melanoma cancer cells. Treatment of melanoma cells with CAP plasma shows promising results in decreasing cell viability and inducing cell apoptosis. The migration ability of melanoma cells is inhibited by CAP plasma treatment and further suppress melanoma cancer cell metastasis. And also the results show that CAP plasma treatment do not have harmful effect on L929 fibroblast cell, but do have obviously suppressed the growth of B16/F10 melanoma cell.
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References


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