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Assessment of Direct and Fluid-Mediated Cold Atmospheric Plasma Treatment Efficacy on Squamous Cell Carcinoma

Gizem Dilara EKİMCİ, Günnur ONAK
Department of Biomedical Engineering
İzmir Katip Çelebi University
İzmir, Turkey
gizemdilara.ekimci@ikc.edu.tr, gunnur.onak@ikc.edu.tr

Ozan KARAMAN and Utku Kürşat ERCAN
Department of Biomedical Engineering
İzmir Katip Çelebi University
İzmir, Turkey
utkuk.ercan@ikc.edu.tr, ozan.karaman@ikc.edu.tr

***Abstract*— Squamous cell carcinoma (SCC) is the second most common skin cancer among the white race. Plasma is an ionized gaseous state of matter containing chemically active species, such as ions, electrons, photons, reactive oxygen and nitrogen species, and UV light. Cold atmospheric plasma (CAP) has just recently been showing promising anti-cancer activities supported by the ability to induce cell death via apoptosis and cell cycle arrest leading to tumor cell destruction *in vitro* and *in vivo*. In this study, two different plasma treatment methods, which are direct plasma treatment and fluid-mediated plasma treatment, apply on SCC and keratinocytes cell lines to determine lethal dose. Also, apoptotic behaviors of two cell types are evaluated with TiterTACS™ apoptosis detection kit. For direct plasma treatment, 60 seconds exposure to CAP found as optimum time and, for fluid-mediated plasma treatment 15 minutes holding of 30 seconds CAP exposure N-Acetyl Cysteine (NAC) solution found as optimum treatment time. Results show that CAP can selectively inactivate SCC cell line through apoptosis while no damage or apoptotic behavior observing in keratinocyte cell line.**

Keywords—Non-thermal plasma, Plasma medicine, Squamous cell carcinoma, Cancer, Biomedical engineering.

I. INTRODUCTION

SCC is the second most seen type of non-melanoma skin cancers after basal cell carcinoma [1]. Prevalence of non-melanoma skin cancer increases rapidly and it is expected to account for 50% of all cancer types in the near future [2]. In the United States, more than one million people are diagnosed with non-melanoma skin cancer annually and 20-30% of those cases are classified as SCC. Annual direct and indirect costs due to non-melanoma skin cancer in the United States are reported as 1.45 billion and 961 million dollars respectively [3]. SCC is the second most seen cancer type among white race and takes more attention and carries more importance due to its higher risk of metastasis compared to basal cell carcinoma [4]. Even though various methods for the treatment of SCC are being used currently, due of parameters such as the phase of disease and comorbidities of the patient, those methods may either fall short or cause serious adverse effects [5].

Plasma is defined as the fourth state of matter and non-thermal plasma can be produced at atmospheric pressure under high electrical field [6, 7]. Different biomedical applications of non-thermal plasma such as coagulation, tooth whitening, disinfection, wound healing, have been reported [8]. Selective efficacy of non-thermal plasma on cancer cell lines by inducing apoptosis and without damaging healthy cell lines have also been demonstrated [9]. Moreover, parallel results to those obtained from cell lines, are shown that plasma treatment of tumors in animal models leads to significant reduction in the weight and volume of tumors [6, 10]. Also, it was demonstrated that liquids, which are treated by non-thermal plasma may gain particular activity similar to the activity plasma treatment shows by itself [11].

The aim of the present study was to evaluate the efficacy of direct and fluid-mediated plasma treatment by the treating N-Acetyl cysteine solution on cutaneous SCC cell line. In the scope of the given study, demonstration of selective effects of direct and fluid-mediated plasma treatment methods on healthy keratinocyte and SCC cell lines were mapped out. Besides, apoptotic behavior of two cell lines investigated.

II. MATERIALS AND METHODS

A. Determination of Lethal Dose on Cells of Different Plasma Treatment Methods

In order to determine the lethal dose, direct plasma application trials were carried out. Direct plasma treatment of cells in the 6-well culture dish is shown in Fig. 1. While inactivating the SCC cell line, several optimization studies have been conducted to determine the parameters of direct plasma treatment with minimal effect on keratinocyte cells.

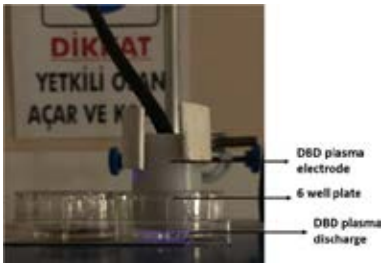

Fig. 1. Direct plasma treatment of cells at 17 kV voltage and 1 kHz frequency using Dielectric Barrier Discharge (DBD) plasma electrode designed and produced within the scope of the study

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As a result of the optimization studies, the viability of the keratinocyte and SCC cells after the 0, 24, 48 and 72 hours post-plasma incubation times and the viability of two cells were compared using standard MTT assay. Direct plasma treatment parameters were 45, 60, 90 and 120 seconds at a voltage of 17 kV and 1 kHz frequency. In these studies, paclitaxel, and non-plasma treated cells were used as control groups.

The paclitaxel used in the studies was provided at a concentration of 6 mg/mL and 100 nM stock solution was prepared using Dulbecco's Modified Eagle Medium (DMEM) broth and stored at -20°C until use. When using, it was used by diluting 2 nM concentration in DMEM medium and adding to 500 µL over the cells.

For fluid-mediated plasma treatment, a liquid holding chamber and a plasma electrode suitably manufactured for this study are used. For this purpose, NAC solution at a concentration of 5 mM was used. To prepare a 5 mM NAC solution, a stock NAC solution at a concentration of 100 mM was first prepared. Therefore, the appropriate amount of weighed NAC powder was dissolved in sterile 1X Phosphate-buffered saline (PBS) solution to a concentration of 100 mM and then subjected to filter sterilization using a 0.22 µm filter and then transferred to 1 mL microcentrifuge tubes and preserved until use at -20°C. When the NAC solution had to be used, the 100 mM stock NAC solution was diluted 20-fold using sterile 1X PBS solution to yield a 5 mM concentration of NAC solution. The process of fluid-mediated plasma treatment is shown in Fig. 2.

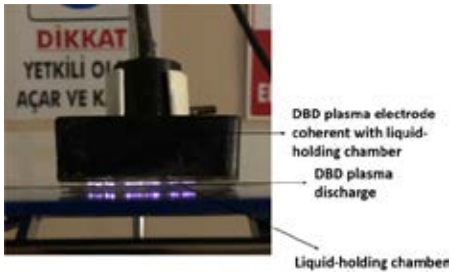

Fig. 2. Fluid-mediated plasma treatment

As a result of a series of optimization studies to determine the plasma treatment time, plasma treatment with 17 kV and 1 kHz parameters, 500 µL of plasma-treated NAC solution for different periods of time by first holding on the cells for 30 minutes, after this trial, viability is 50% and lower so, plasma-treated NAC solution was maintained on the cells for 15 minutes.

B. Investigation of the Effectiveness of Different Plasma Treatment Methods on Apoptosis

Following the plasma treatment of healthy keratinocyte and SCC cell lines using the plasma parameters optimized for the experiments, apoptotic behavior of the cells at 0, 24, 48 and 72 hours have been identified following the manufacturer's recommended procedure using the TiterTACS™ apoptosis kit.

At specified times following plasma treatment with the different plasma treatment parameters determined, the cells were removed by trypsinization on the surface and incubated in 3.7% buffered formaldehyde solution. After washing step, 100% methanol was added to the cells to incubate for 20 minutes. Subsequently, labeling was carried out. The cells were incubated by adding Proteinase K and then, for inactivation of endogenous peroxidase, 2.5% H_2O_2 was added to each well and incubated. Subsequently, 1X TdT labeling buffer and after that labeling reaction mixture was added to each well and incubated. After incubation, reaction stop buffer was added to each well incubated. After washing, Streptavidin-HRP solution added and incubated. Then, Streptavidin-HRP solution of TACS-Sapphire was added per well and incubated for 30 minutes at room temperature and in the dark. After incubation, 2N HCl was added to stop the reaction and the absorbance was measured at 450 nm. Non-plasma treated cells and cells exposed to paclitaxel were used as controls. In addition, apoptotic cells were examined under the microscope and their images were taken after plasma treatment.

All experiments were performed in triplicate for each condition and repeated at least twice. In showing the results, the control groups were normalized to 100% and the other experimental groups were presented as a percentage increase and decrease.

III. Results and Discussions

A. Determination of Lethal Dose on Cells of Different Plasma Treatment Methods

As a result of the studies conducted to determine the lethal dose of different plasma treatment methods on cells, approximately 90% inactivation of SCC cells was determined at the end of each 45, 60, 90 and 120 seconds plasma treatment period, and the viability rates after 24 hours plasma incubation for keratinocyte cells were determined 81.8, 94.1, 72 and 52.6 respectively (Fig. 3). The viability of keratinocytes was increased for each plasma treatment time after 48 and 72 hours of post-plasma incubation, whereas this increase was not observed in SCC cells. Besides, when the viability trends of keratinocyte cells and SCC cells are examined, it is observed that 60 seconds of direct plasma treatment is the optimum time.

Also, the viability of keratinocyte and SCC cells at different incubation times after each direct plasma treatment when the paclitaxel was used as a control group, the determined plasma treatment parameters effectively inactivated the SCC cells and did not cause the inactivation of the keratinocytes.

Although fluid-mediated plasma application revealed 50% viability after 24 hours incubation, which 15 minutes of exposure of cells to NAC solution treated for 30 seconds in plasma treatment, it was observed that this viability increased to 75% after 48 hours and 72 hours incubation. Likewise, in the application to SCC cells, it was determined that the viability around 73% after 24 hours and decreased to 18% after 72 hours incubation. In paclitaxel treatment, viability was observed to be 10% after 24, 48 and 72 hours incubation for each cell type (Fig. 4).

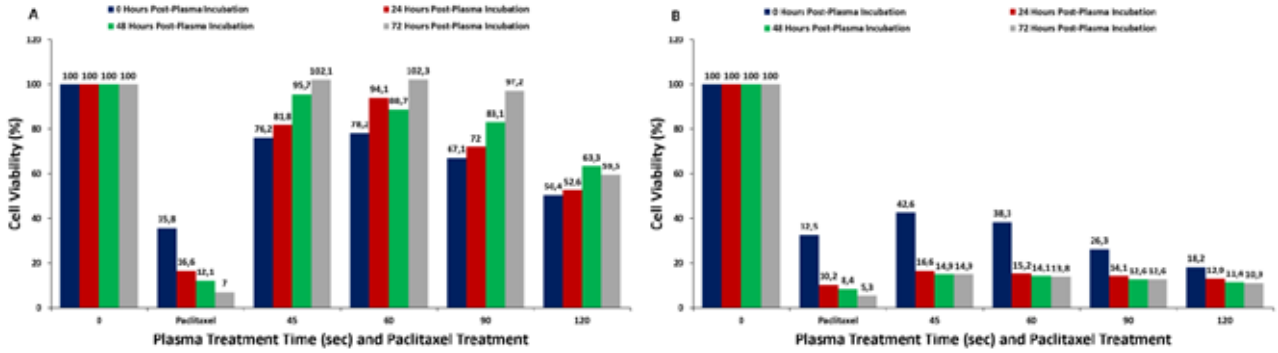

Fig. 3. Viability values of keratinocyte (a) and SCC (b) cells obtained after direct plasma treatment for 45, 60, 90 and 120 seconds as a result of determined plasma parameters

Besides, when the viability of keratinocyte and SCC cells at different incubation times after each fluid-mediated plasma treatment parameters, which paclitaxel was used as a control group (Fig. 4), it is outstanding that 15 minutes exposure to 30 seconds fluid-mediated plasma-treated NAC solution was the optimum time.

B. Investigation of the Effectiveness of Different Plasma Treatment Methods on Apoptosis

As a result of the studies conducted to investigate the efficacy of different plasma treatment methods on apoptotic behavior, it was observed that apoptotic SCC cells increased with the increasing post-plasma incubation time at the end of fluid-mediated and direct plasma treatment times. Besides, to determine apoptotic behavior for keratinocyte cells, it was observed that apoptotic cells did not change over post-plasma incubation time. Also, it was found that the determined plasma treatment parameters caused apoptosis in SCC cells and did not cause apoptosis in keratinocyte cells. It has also been observed that direct plasma treatment of SCC cells results has more apoptotic behavior than fluid-mediated plasma treatment (Fig. 5).

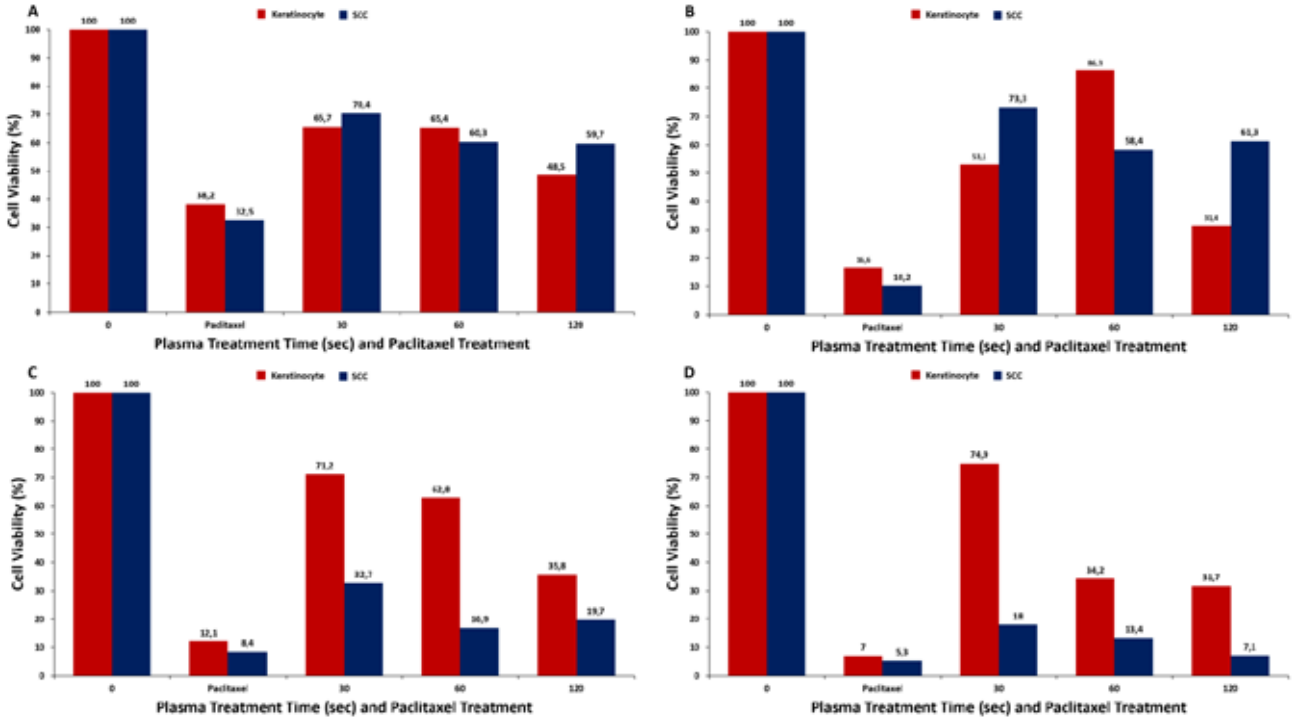

Fig. 4. Comparing the viability between the keratinocyte and SCC cells holding for 15 minutes after the plasma treatment of NAC solution which was subjected to plasma treatment for 30, 60 and 120 seconds (a): 0, (b): 24, (c): 48 and (d): 72 hours incubation periods

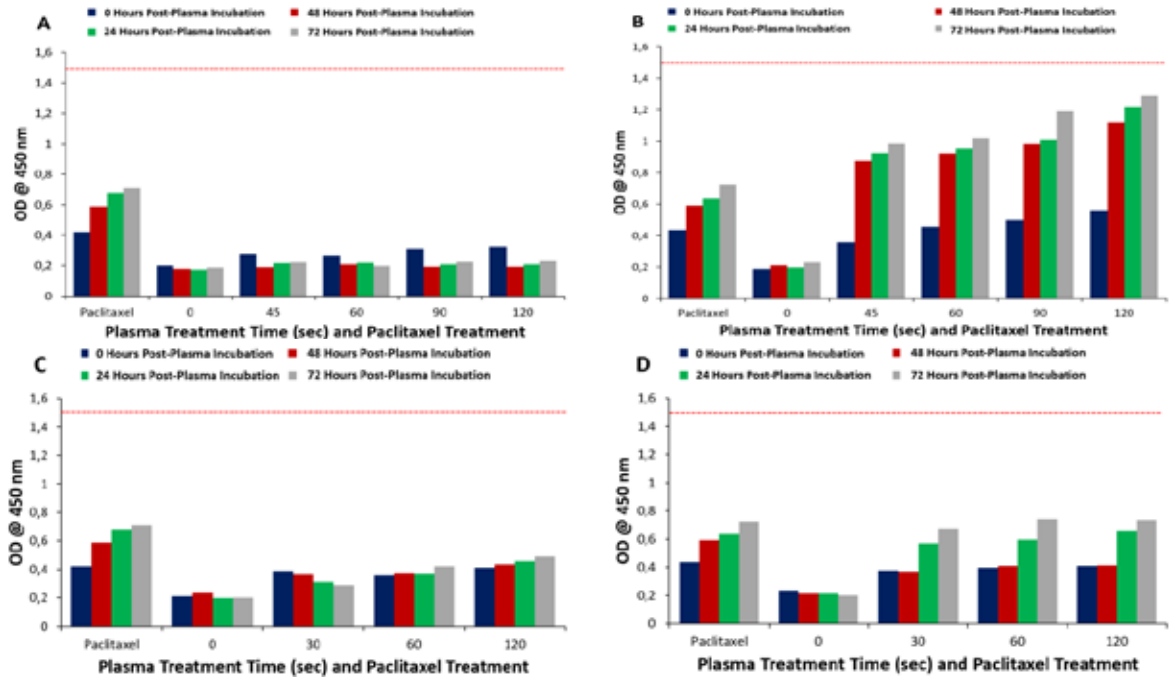

Fig. 5. Absorbance readings at 450 nm using the TiterTACS™ kit in keratinocytes and SCC cells treated with plasma with different plasma treatment parameters. (a): direct plasma treatment of keratinocytes; (b): direct plasma treatment of SCC cells; (c) fluid-mediated plasma treatment of keratinocytes; and (d) fluid-mediated plasma treatment of SCC cells. The red dashed line shows the absorbance reading from nuclease-treated cells

In addition to the aforementioned studies, the change in cell morphology obtained during the studies carried out suggested that different plasma treatment methods caused apoptosis in the cells, and the photographs in which these morphologies were shown in Fig. 6.

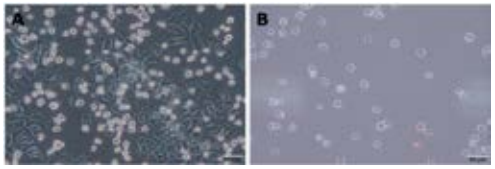

Fig. 6. Cell morphology suggesting apoptosis in photographs obtained from studies. (a): cell morphologies suggesting both viable and apoptosis in keratinocytes by direct plasma treatment of 120 seconds; and (b): SCC cells suggesting apoptotic morphology by direct plasma treatment of 120 seconds

IV. Conclusions

In this study, the viability and apoptosis efficiencies of healthy keratinocyte and SCC cell lines as a result of fluid-mediated and direct plasma treatment were evaluated. Plasma treatment showed low levels of viability in the SCC cell line, while high levels of viability were observed in the keratinocyte cell line. Similarly, high levels of apoptosis were observed in SCC cell line compared to the keratinocyte cell line as a result of studies to determine apoptosis. Atmospheric cold plasma treatment showed a selective inactivating effect on SCC, but no damage to keratinocyte cells. Successful completion of the proposed study may be considered as an important step for the utilization of various plasma treatment methods as a novel tool for the treatment of SCC.

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