Short Communication

Combined Anticancer Effect of Treatment with Cold Atmospheric Plasma and Thymidine

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Abstract

Objective: In recent years, the anticancer effect induced by cold atmospheric plasma (CAP) has received much attention and has become a rapidly growing research topic. The purpose of this article is to study the anticancer effect of CAP combined with drugs to seek more efficient anticancer therapy.

Methods: The plasma source used in this study was surface air discharge plasma, and the anticancer drug was thymidine (TMD). Breast cancer cells (MCF-7) and cervical cancer cells (HeLa) were treated in vitro to evaluate the anticancer effect of the combination of the two therapies. The anticancer efficiency was assessed by detecting cell viability and cell apoptosis rate. And the combined anticancer mechanism was further analyzed by measuring intracellular reactive oxygen species (ROS) levels.

Results: The results of cell viability and cell apoptosis showed that the combined anticancer effect of plasma and TMD was significantly better than TMD treatment alone, but not as good as plasma treatment alone. Intracellular ROS levels were increased by plasma treatment of MCF-7 and HeLa cells, and ROS played an important role in plasma-induced apoptosis of cancer cells. In addition, TMD didn’t induce intracellular ROS production, but only interfered with DNA replication and normal division of cancer cells and could increase the stress of cell division, thus inducing apoptosis of cancer cells.

Conclusion: TMD may interfere with the targets of plasma action and affect the anticancer effects of plasma, which indicated that not all anticancer drugs can achieve synergistic effect with plasma, and some drugs even interfere with plasma action.

Keywords: cold atmospheric plasma, thymidine, combined anticancer effect, reactive oxygen species, apoptosis
1 INTRODUCTION
In recent years, there has been significant interest in the use of cold atmospheric plasma (CAP) for various biomedical applications, including disinfection, wound healing, and cancer therapy. Numerous studies have demonstrated that CAP can effectively induce apoptosis in a variety of cancer cells, such as melanoma, lung cancer, cervical cancer, and breast cancer. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by CAP have been reported to play a key role in the induction of apoptosis in cancer cells. Gas-phase reactive species produced by CAP can react with the pericellular medium to form large amounts of aqueous ROS and RNS. It is generally accepted that the main intermediates for plasma-cell interactions are aqueous ROS and RNS. In addition, plasma-activated liquid such as plasma-activated water or plasma-activated medium can act on cancer cells for a long time due to the presence of various reactive species, which makes it possible to treat tumors deep in the body by precise injection in clinical applications.

Breast and cervical cancers are the most common malignancies in females. According to the American Cancer Society’s 2016 cancer statistics, breast cancer accounts for approximately 29.2% of all female malignancies and cervical cancer accounts for approximately 8.7%, and they rank first and third respectively. Currently, surgical techniques, drug therapy, and radiation-based approaches are the primary methods of clinical cancer treatment. However, radiotherapy can severely damage the body and drug therapy can make cancer cells resistant to treatment. In recent years, several studies have considered combining plasma and traditional clinical drugs. It has been found that the combination of plasma and low-dose drugs has a synergistic effect on cancer treatment. However, different types of clinical drugs have different therapeutic mechanisms for tumors, and their combination with plasma has different effects. The study of synergistic mechanisms can help accelerate the clinical application of CAP in cancer treatment.

In this paper, HeLa cells and MCF-7 cells were selected as treatment targets. The cancer cells were treated in vitro by combining CAP and thymidine (TMD), an anticancer drug that induces apoptosis by interfering with DNA replication and normal division of cancer cells. The anticancer effect of CAP in combination with TMD was explored by measuring cell viability and apoptosis rate. In addition, the underlying mechanism of the anticancer effect was further discussed by analysis of the ROS accumulation after different treatments.

2 EXPERIMENTAL METHODS
2.1 The Experimental Setup
Figure 1A depicted the experimental configuration for the surface air discharge plasma. Plasma was generated by a sandwich-like dielectric barrier discharge device consisting of a copper high-voltage electrode, a mesh ground electrode, and an aluminum oxide ceramic insulating dielectric layer. As seen in Figure 1B, the plasma source was driven by a sinusoidal voltage of 6kV (peak to peak) at a frequency of 30kHz. The voltage and discharge current waveforms were measured by an oscilloscope (Tektronix, DPO3000) with a high-voltage probe (Tektronix, P6015A) and a current probe (Pearson, 2877). A Fourier transform infrared spectrometer (Bruker, Tensor II) was used to detect the gaseous reactive species in the wavenumber from 500 to 2500cm⁻¹. As shown in Figure 1C, the gaseous reactive species generated by surface air discharge plasma are mainly N₂O, NO₂, and N₂O₃. The plasma treatment process of cancer cells is shown in Figure 1A. The distance between plasma and the surface of cell culture medium was 5mm, and the cancer cells were put in petri dishes of 35*10mm with 3mL of cell culture medium with the density of 2×10⁵ cells/dish.

2.2 Cell Culture
In this study, human cervical carcinoma cell line and breast carcinoma cell line were purchased from the American Type Culture Center (Manassas, USA). Both two cell lines were cultured in DMEM (Invitrogen Life Technologies, USA) containing 10% fetal calf serum (Thermo Scientific, Rockford, USA) and 1% penicillin-streptomycin (100×, Med. Chem. Express, USA). The cells were cultured at an incubator with 95% air and 5% CO₂ at constant temperature of 37°C. Cells were harvested via detachment with a combination of trypsin and ethylenediaminetetraacetic acid (0.25%, 0.02%).

2.3 Cell Viability and Apoptosis Assays
Cell viability was detected by a microplate reader (Thermo Scientific Varioskan® Flash, USA) with Cell Counting Kit-8 (CCK-8) assay (Beyotime Biotech, China). Cell apoptosis was accessed by the flow cytometry (Beckman Coulter, Fullerton, CA) with an Annexin V-FITC/PI staining detection kit (BD Biosciences, USA) per the manufacturer's instructions.

2.4 Quantification of Oxidation-reduction Potential (ORP) and ROS in Cell Culture Medium
The ORP values of cell culture medium were detected by an ORP electrode (Mettler Toledo, USA). The ROS (H₂O₂, NO₂⁻ and NO₃⁻) concentrations in cell culture medium were detected by a microplate reader (Thermo Scientific
Varioskan® Flash, USA) using Hydrogen Peroxide Assay Kit, Griess Reagent Kit, and Total Nitric Oxide Assay Kit (Beyotime, China), respectively. And the concentrations of O$_3$ were detected by using O$_3$ AccuVac® Ampoule Reagent (Hach, USA) with a spectrophotometer (Hach, USA).

2.5 Detection of Intracellular ROS

The ROS assay kit (Beyotime Biotech, China) was used to measure intracellular ROS of treated Hela and MCF-7 cells as per instructions. The cells were cultured with 10µM DCFH-DA for 25min at 37°C after treated and then the ROS level was detected by flow cytometry (Accuri C6, BDBiosciences, Bedford, MA, USA).

2.6 Statistical Analysis

All experimental were performed three times and data were presented as the mean±SD and analyzed by *t*-test method. *P*<0.05 was considered statistically significant (*P*<0.05, **P*<0.01).

3 RESULTS AND DISCUSSION

3.1 Effects of Plasma, TMD, and Combination of Them on Cell Morphology

The effects of plasma and TMD on cell morphology are shown in Figure 2A. Here 10µM TMD (Beyotime Biotechnology, China) drug dose was selected for the whole experiments. The plasma treatment time was 20min. The steps of combined treatment were as follows: (1) TMD was added into cell culture medium with the concentration of 10µM; (2) the cell culture medium with added TMD was exposed under the plasma for 20min; (3) the prepared cell culture medium then incubation with HeLa and MCF-7 cells for 24h. The pictures were taken by a microscope after 24h of cell incubation. As shown in Figure 2A, after 24h of drug treatment alone, there was no obvious change in the morphology of HeLa cells and MCF-7 cells and only a small number of cells floated in the culture solution. After 24h of plasma treatment alone, cancer cells became rounded and shrunk, and the connection between them was reduced. Moreover, many cancer cells are apoptotic and floated in the medium. For the combined treatment, the results were better than that of drug treatment alone, but not as effective as plasma treatment alone. It indicated that the TMD seemed to weaken the effect of the plasma.

3.2 Effects of Plasma, TMD, and Combination of Them on Cell Viability and Apoptosis

The cell viability was examined to verify our conjecture, and the results are presented in Figure 2B. A decrease in cell viability of both HeLa and MCF-7 cells was seen regardless of experimental methods. In detail, the
Figure 2. Effects of plasma, TMD and combination of them on cell morphology, cell viability, apoptosis and ROS accumulation. A: The morphological change in HeLa and MCF-7 cells after TMD treatment, plasma treatment and combined treatment; B: The cell viability detection of HeLa cells and MCF-7 cells after TMD treatment, plasma treatment and combined treatment; C, D: The apoptosis rate detection and apoptosis rate statistics of HeLa and MCF-7 cells after TMD treatment, plasma treatment and combined treatment; E: The ORP values and the concentration of $O_3$, $H_2O_2$, $NO_2^-$ and $NO_3^-$ after TMD treatment, plasma treatment and combined treatment; F: The intracellular ROS levels detection after TMD treatment, plasma treatment and combined treatment.

cell viability decreased with extending incubation time and increasing treatment time. As shown in Figure 2B, the most efficient treatment was plasma treatment alone. After treatment with plasma for 20min and incubation for 24h, the cell viability of HeLa / MCF-7 cells were reduced to 4.2%/3.7%, compared to 93.5%/87.5% with drug treatment alone and 32.5%/9.42% with combined treatment. In summary, the efficiency of plasma treatment alone is higher than that of the combined treatment and the efficiency of the combined treatment is higher than that of the drug treatment alone.

To further analyze the differences of treatment efficiency, the apoptosis rates of HeLa and MCF-7 cells under different treatment methods were studied. As shown in Figure 2C and 2D, for HeLa / MCF-7 cells, the apoptosis rate increased to 8.3%/14.9% with drug treatment alone, 62.1%/76% with plasma treatment alone, and 42.3%/49.4% with combined treatment. The results were consistent with the results of the cell viability examination: the anticancer effect of combined treatment was significantly better than the TMD treatment alone, but less effective than plasma treatment alone.

For the above experimental results, two conjectures are given here: (1) TMD may react with ROS in the cell culture medium or reduce the intracellular ROS level, thus allowing cancer cells to cope with the higher oxidative stress caused by ROS from plasma; (2) TMD may arrest cancer cells in S-phase, and it is possible that plasma treatment has
a weaker effect on S-phase cancer cells. For these two conjectures, the relevant experiments and analysis are as follows.

3.2 Effects of Plasma, TMD, and Combination of Them on ROS Accumulation

The oxidative pressure on cancer cells was determined by the intracellular ROS levels and the concentrations of ROS in the cell culture medium. Studies showed that plasma can increase the intracellular ROS levels by causing the accumulation of ROS in cell culture medium, and then induce apoptosis\(^\text{[12]}\). Here, the ORP values of cell culture medium were detected, as shown in Figure 2E. The ORP value of drug treatment alone did not change significantly compared to the control group. For both plasma treatment alone and combined treatment, they caused an increase of ORP values. However, the ORP values of plasma treatment and combined treatment were almost same, which indicated that TMD did not affect the accumulation of plasma-induced aqueous ROS. Then, the ROS concentrations of cell culture medium after plasma treatment were detected, as shown in Figure 2E. The ROS concentrations increased gradually with plasma treatment time and the concentrations of O$_2$, H$_2$O$_2$, NO$_2^-$, and NO$_3^-$ were 2.7mg/L, 46.5mg/L, 119.6mg/L, and 151.9mg/L after treatment for 20min.

Figure 2F showed the detection results of intracellular ROS levels. The drug treatment alone did not cause an increase in intracellular ROS levels compared to the control group. Both plasma treatment and combined treatment caused an increase in intracellular ROS levels, which increased with the treatment time. There was little difference in intracellular ROS levels after plasma treatment alone and combined treatment, indicating that TMD did not affect plasma-induced intracellular ROS accumulation. To sum up, TMD had no effect on the accumulation of intracellular ROS and concentrations of ROS in cell culture medium. The results indicated that the interference of TMD on the plasma treatment effect was achieved by other ways.

In recent years, researchers found that the distribution of cell cycles changed after plasma treatment by plasma-induced cell cycle arrest, which resulting proliferation stress of cancer cells and then caused apoptosis\(^\text{[13-14]}\). So cancer cells in different phases may have different sensitivity to plasma. TMD can make cancer cells stay in the S-phase, and if G$_2$/M phase cells are more sensitive to plasma than S phase cells, TMD would interfere with the effect of plasma on cancer cells, thereby reducing the anticancer effect of plasma. In order to get a good therapeutic effect, anticancer drugs need to arrest the cancer cells in the most sensitive cell phase to plasma before plasma treatment. The accurate mechanism of combined treatment with plasma and anticancer drugs still needs further study in the future.

4 CONCLUSION

In this work, combined anticancer effect of treatment with CAP and TMD was studied. By comparing the cell morphology, cell viability and apoptosis of Hela cells and MCF-7 cells, it was found that the anticancer effect of combined treatment with plasma and TMD was less effective than plasma treatment alone, but significantly better than the TMD treatment alone. Further research indicated that TMD had no effect on the accumulation of intracellular ROS and concentrations of liquid-phase ROS after plasma treatment. We hypothesized that TMD interferes with the target of plasma by stalling cancer cells in S phase, thus weakening the anticancer effect of plasma.

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Conflicts of Interest

The authors have no conflict to disclose.

Author Contribution

Jing X investigated, collected, and analyzed the data and wrote the original draft. Xu S was involved in methodology and supervision as well as reviewed and edited the article. Zhang H, Xu H and Liu D reviewed and edited the article. All authors discussed the results and commented on the paper.

Abbreviation List

CAP, Cold atmospheric plasma
ORP, Oxidation-reduction potential
RNS, Reactive nitrogen species
ROS, Reactive oxygen species
TMD, Thymidine

References


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