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Anticancer Efficacy of Long-Term Stored Plasma-Activated Medium

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Abstract: The therapeutic potential of nonthermal atmospheric-pressure plasma for cancer treatment via generation of reactive species, induction of decreased mitochondrial membrane potential, and sequential apoptosis has been reported in our previous studies. Nonthermal atmospheric-pressure plasma-activated medium produced by jetting air plasma above a liquid surface shows advantages over direct plasma such as storage and delivery to tissues inside the body. In this study, we demonstrated that plasma-activated medium can be stored for up to 6 months in a freezer and that the stored plasma-activated medium has anticancer effects similar to those of direct plasma. Plasma-activated medium stored for 6 months showed cytocidal effects on human cervical cancer HeLa cells that were comparable to the effects of fresh plasma-activated medium or direct plasma. Furthermore, the levels of reactive species in plasma-activated medium persisted for up to 6 months. These results indicate that therapeutic application of plasma-activated medium is applicable in plasma medicine and is a promising anticancer strategy.

Keywords: plasma-activated medium; reactive oxygen species; apoptosis

1. Introduction

The fourth state of matter, plasma, is a partially ionized gas containing a high density of electrons and various reactive radicals and non-radicals. Recently, to enable the use of plasma in biomedical applications, various types of low-temperature atmospheric-pressure plasmas have been studied [1–6]. Dielectric barrier discharge is most often used to generate plasma because of its high electron density and insulation ability [4].

Cancer is among the leading causes of mortality worldwide and the treatment of patients with metastatic or relapsing cancer represents a main therapeutic challenge. New approaches to cancer therapy may involve the use of plasma. Biomedical applications of plasma have been demonstrated in a variety of experiments [5–11]. Recently, studies have revealed the ability of atmospheric-pressure plasma to selectively eliminate cancer cells, indicating the potential of nonthermal atmospheric-pressure plasma as an anticancer agent [1,3,7–10]. Nonthermal atmospheric-pressure plasma appears to be safe in the human body and normal cells but shows anticancer activity [1,3,7–10]. In contrast, plasma treatment may induce the death of normal primary prostate epithelial cells via DNA damage-mediated necrosis [11]. The selective and non-selective actions of plasma may occur through its dose-dependent differential effects on cancer and normal cells or cell type-dependent cytotoxic effects. Therefore, additional studies are needed to validate

the potential therapeutic use of nonthermal atmospheric-pressure plasma, particularly against cancer. We and many groups have reported various characteristics of atmospheric-pressure plasma, such as induction of apoptosis in cancer cells via DNA damage, mitochondrial collapse, and aberrations in the cellular membrane [1–3,5,9,12–19]. Additionally, recent studies revealed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by atmospheric-pressure plasma play crucial roles in inducing cancer cell death [1,3,10,15,16,19].

However, atmospheric-pressure plasma has two main limitations in biomedical applications. One is the limited penetration of plasma into tissues, the other is storage difficulties. To overcome these limitations, we employed plasma-activated medium (PAM) generated with nonthermal atmospheric-pressure plasma [3,8,20–25]. We evaluated the anticancer efficacy and storage feasibility of the PAM.

2. Materials and Methods

2.1. Reagents and Antibodies

We used the following reagents: fluorescein isothiocyanate (FITC) Annexin V Apoptosis Kit I (BD Pharmingen, Franklin Lakes, NJ, USA); Amplex UltraRed Kit (Life Technologies, Carlsbad, CA, USA); Griess Kit (Life Technologies); Live and dead cell assay Kit (Life Technologies); and MTT assay Kit (Abcam, Cambridge, UK).

2.2. Micro Plasma-Jet Nozzle

The structure of the micro plasma-jet nozzle for the nonthermal atmospheric-pressure plasma jet is shown in Figure 1a. The nozzle consists of an anode, stainless-steel electrode ring, and cathode. The anode was a 100-μm-thick nickel-cobalt layer on a 500-μm-thick glass wafer. The anode contained 25 holes with a diameter of 300 μm. The pattern of the hole arrangement was described previously [26]. The cathode was a stainless-steel tube through which gas was supplied and the inner diameter of the tube was 1.5 mm. The anode was fabricated by photolithography and nickel-cobalt electroplating with a Cr/Au seed layer on a glass wafer. After chemical mechanical polishing of the glass wafer, the anode holes were fabricated by sand blasting. These electrodes were packaged in a plastic case.

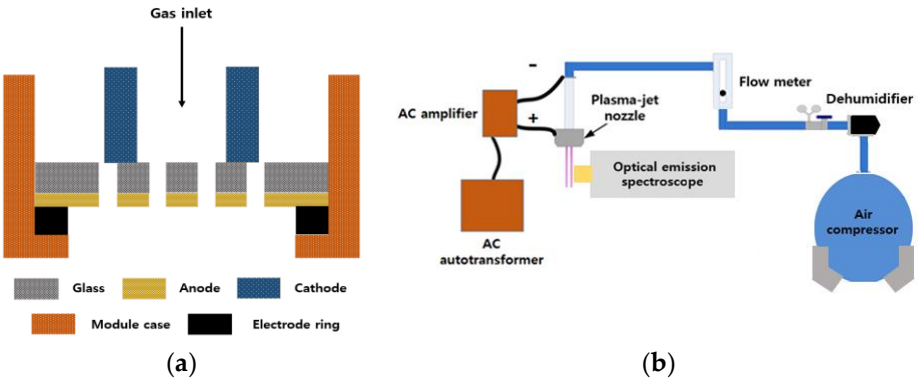


Figure 1. Nonthermal atmospheric-pressure plasma-jet system. (**a**) Structure of the micro plasma-jet nozzle. (**b**) Experimental set-up for discharge experiments.

2.3. Nonthermal Atmospheric-Pressure Plasma-Jet System

Figure 1b shows the experimental set-up for the discharge experiments using the nonthermal atmospheric-pressure plasma-jet system. The power controller was a variable AC autotransformer supplying 15 kV_{p-p} at a frequency of 20 kHz. Air was supplied through the tube at 10 L/min. We recorded the optical emission spectrum of the plasma jet using an optical emission spectroscope (SV 2100, K-MAC, Daejeon, Korea, 2012) to identify radicals useful for biomedical applications.

2.4. Preparation of Plasma-Activated Medium (PAM)

As shown in Figure 2, using this air plasma-jet system, we jetted nonthermal air plasma 2 cm above the surface of Dulbecco's Modified Eagle Medium (DMEM) (WELGENE, Daejeon, Korea) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies), a mammalian cell culture medium, in a chamber of a 12-well-plate for 5 min at atmospheric pressure and room temperature to generate PAM. The height and diameter of the well chamber were 2.5 and 2.2 cm, respectively. We evaluated the dissolved ozone concentration in the PAM using a waterproof portable colorimeter (C105, EUTECH, Singapore). For the storage test, we stored the PAM in a freezer (−20 °C) for up to 6 months.

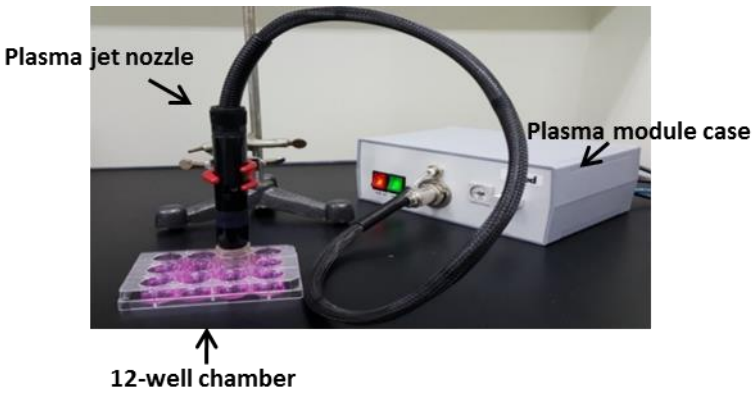


Figure 2. Generation of plasma-activated medium (PAM) using DMEM by jetting air plasma 2 cm above the surface of the medium in a well (2.5 × 2.2 cm) of a 12-well-plate.

2.5. Quantification of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

The concentrations of extracellular H_2O_2 were measured using an Amplex UltraRed hydrogen peroxide assay kit (Invitrogen, Carlsbad, CA, USA). Florescence intensity was measured on a microplate reader (Bio-Rad, Hercules, CA, USA) at 530/590 nm according to the manufacturer's protocol. The relative ROS level was calculated in terms of arbitrary fluorescence units. The production of RNS in the culture supernatant by plasma was detected as described previously using a Griess assay kit (Invitrogen).

2.6. Cell Culture and Treatment with PAM

Human cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and antibiotics. All cells were maintained at 37 °C in a humidified incubator at 5% CO_2. Cells (approximately 5×10^4) were seeded into 12-well plates with 1 mL of DMEM supplemented with 10% of FBS and grown overnight before treatment with the fresh/stock PAM. The cells were washed with Dulbecco's phosphate-buffered saline (Life Technologies) and covered with either the fresh/stock gas-treated DMEM or fresh/stock PAM. Next, the cells were incubated at 37 °C for 24 h to assess the anticancer efficacy of the PAM.

2.7. Detection of Apoptosis

During treatment with PAM, the cells were incubated at 37 °C in a humidified incubator at 5% CO_2 for 24 h. The cells were harvested with trypsin-EDTA and rinsed with phosphate-buffered saline. To detect plasma-induced apoptosis, we stained the cells with FITC-conjugated anti-annexin V or propidium iodide (Invitrogen). After staining, the cells were analyzed by flow cytometry (BD FACSAria III).

2.8. Measurement of Cellular Viability

After 24 h of treatment with either the fresh/stock PAM or direct plasma, the live/dead assay involved double-labeling of the cells with 2 μM calcein AM and 4 μM EthD-1 according to the manufacturer's instructions (Life Technologies and Abcam). Calcein AM-positive live cells and EthD-1-positive dead cells were examined under a fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-S/L100, Tokyo, Japan). Additionally, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Abcam) was performed to evaluate cellular viability and activity as previously described [1,19].

2.9. Statistical Analysis

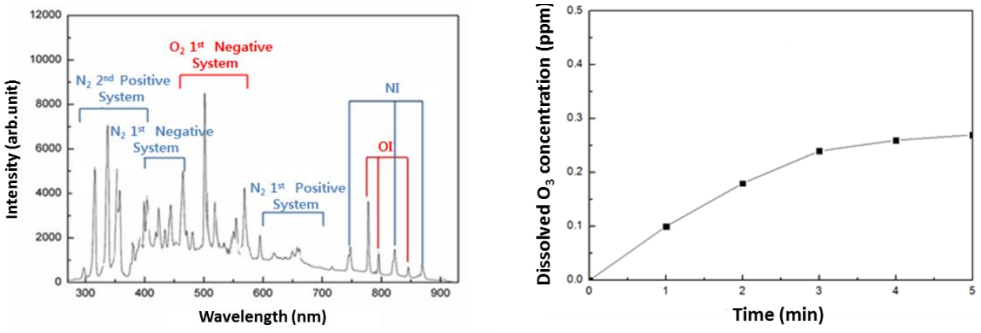
All data were expressed as mean ± standard deviation (SD) of at least three replicates. Student's *t* test was used for the analysis of significance of differences between datasets. Differences were considered statistically significant at $p \leq 0.05$ (in figures: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3. Results

3.1. Reactive Species Generated in Plasma Jet and PAM

Figure 3a shows the optical emission spectrum of the nonthermal atmospheric-pressure air plasma jet in the atmosphere 1 cm apart from the nozzle end over a wide range of wavelengths from 280 to 920 nm. The air plasma contained excited oxygen ions (O_2+) and excited nitrogen molecules as the ROS and RNS, respectively.

Dissolution of the plasma indicates that the plasma-jet system is useful for various biomedical applications [27]. We adopted dissolved ozone among various reactive oxygen species in order to find an appropriate plasma-treatment time. Figure 3b shows that the concentration of dissolved ozone in plasma-treated DMEM increases with increasing plasma-treatment time. Since the concentration reached a saturation level of 0.3 ppm in 5 min, the plasma-treatment time was set to 5 min in the preparation of PAM.



(**a**) (**b**)

Figure 3. Reactive species generated in plasma jet and PAM. (**a**) Optical emission spectrum of air plasma jet. (**b**) Concentration of dissolved ozone in Dulbecco's Modified Eagle Medium (DMEM) vs. plasma treatment time.

3.2. Anticancer Efficacy of PAM

Recent studies revealed that treatment of cancer cells with direct nonthermal plasma induces apoptotic cell death, accompanied by ROS accumulation, reduced mitochondrial membrane potential, and mitochondrial dysfunction [1,19]. To use of plasma in medical applications, there are several essential prerequisites including long-term storage and efficient delivery to internal parts of the human body. Therefore, we first evaluated the anticancer effects of PAM compared to those of direct plasma. After 24 h of incubating the cells with PAM or gas-treated medium or after 24 h of incubating the

cells exposed to direct plasma for 5 min, cell viability assay was conducted by double-labeling of the cells with calcein AM and EthD-1. Calcein AM-positive live cells and EthD-1-positive dead cells were assessed under a fluorescence microscope (Figure 4a). Additionally, cell viability and activity were evaluated by MTT assay after plasma treatment (Figure 4b). PAM caused the death of human cervical cancer HeLa cells, analogously to direct air plasma, while gas treatment had no effects on cancer cell viability (Figure 4). This suggests that PAM has comparable anticancer effects as direct plasma (Figure 4).

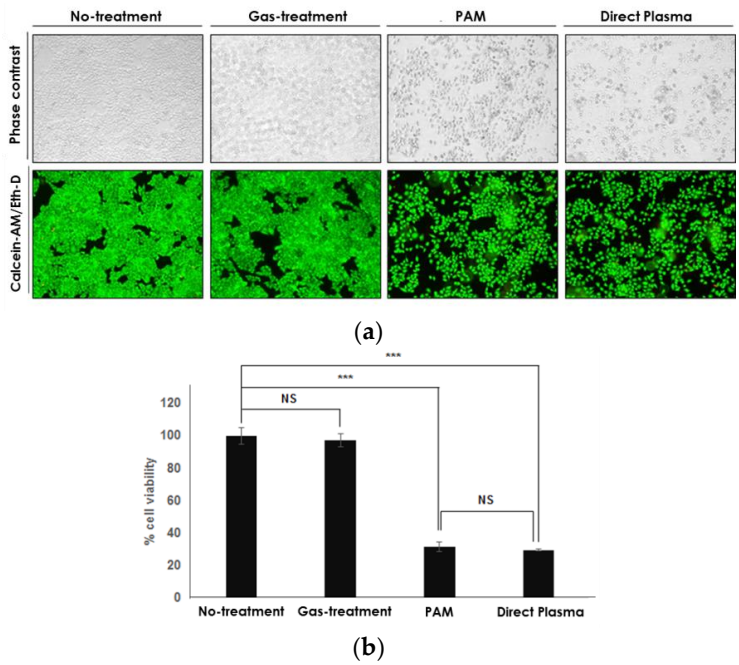


Figure 4. Comparison of anticancer effects of PAM and direct plasma. (**a**) Human cervical HeLa cancer cells were treated with either PAM or direct plasma, or gas (gas-treatment), stained with calcein-AM and EthD-1, and their viability was examined under a fluorescence microscope. (**b**) Cell viability and activity were assessed by MTT assay and compared to those of untreated or gas-treated control cells. NS, not significant; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3. Anticancer Efficacy of Frozen PAM

Next, we evaluated the period of efficacy of PAM. PAM was stored frozen and thawed at the indicated time-points to treat HeLa cancer cells. The results in Figure 5a–b show no significant differences in the effects on HeLa cancer cell viability between fresh and stocked PAM that had been frozen and thawed. The effect was also comparable to that observed for direct plasma treatment. Additionally, we examined whether fresh and stock PAM induced apoptosis in cancer cells after 24 h of treatment with PAM by staining the cells with annexin V conjugated with FITC and PI. After staining, cell death was analyzed by flow cytometry. Apoptosis was detected by labeling phosphatidylserine on the membrane surface of apoptotic cells with annexin V, and late apoptosis/necrosis were detected by labeling cellular DNA with PI. Figure 5c shows that 1- and 6-month stock PAM caused similar levels of cancer cell death and most PAM-treated cancer cells underwent apoptosis.

Further, we quantitatively assessed the anticancer effects of fresh and stock PAM by diluting PAM and then evaluating its cytotoxic effects on HeLa cells in a live/dead cell assay (Figure 6a) and MTT assay (Figure 6b). Dilutions of PAM by half or 1/4 efficiently induced cell death of HeLa cells but caused slightly lower cell death as compared to undiluted fresh and stock PAM (Figure 6). However, 1/10-diluted PAM did not cause cell death of HeLa cells. Thus, we estimated that the anticancer effect of fresh and stock PAM ranged from 1/10 to 1/4 dilutions. Moreover, we observed a similar dilution

effect between the fresh and stock PAM (Figure 6), indicating that the components required for the anticancer effects in the stock PAM do not decline over time.

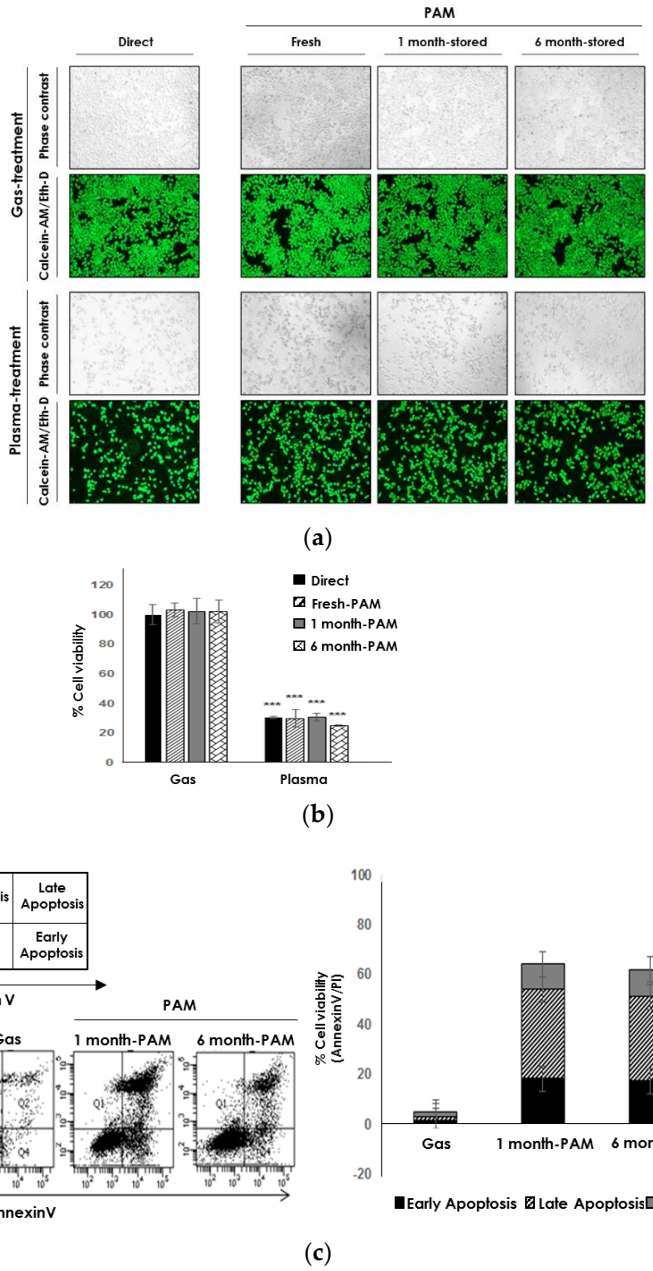


Figure 5. Efficacy of frozen PAM. PAM was stored in a freezer (−20 °C) for the indicated periods (1 and 6 months), then thawed and added to HeLa cells to evaluate its efficacy. (**a**) Human cervical HeLa cancer cells were treated with plasma (direct plasma or fresh and 1- or 6-month stock PAM) (plasma-treatment) or gas (gas or gas-treated fresh and stock medium) (gas-treatment) for 24 h. Cellular viability was assessed using calcein-AM and EthD-1 to detect live and dead cells, respectively. (**b**) Cell viability was measured by MTT assay and compared to those of gas-treated control cells. (**c**) Cell death via apoptosis/necrosis was detected by staining the cells with FITC-conjugated annexin V and propidium iodide (PI), respectively, and by flow cytometric analysis, and compared to that in gas-treated cells. The dot plots show representative images of apoptotic cell death after PAM-treatment, identified by labeling phosphatidylserine on the membrane surface of early/late apoptotic cells with annexin V, and by labeling DNA of late apoptotic/necrotic cells with PI. The graph shows the populations of apoptosis/necrotic cells.

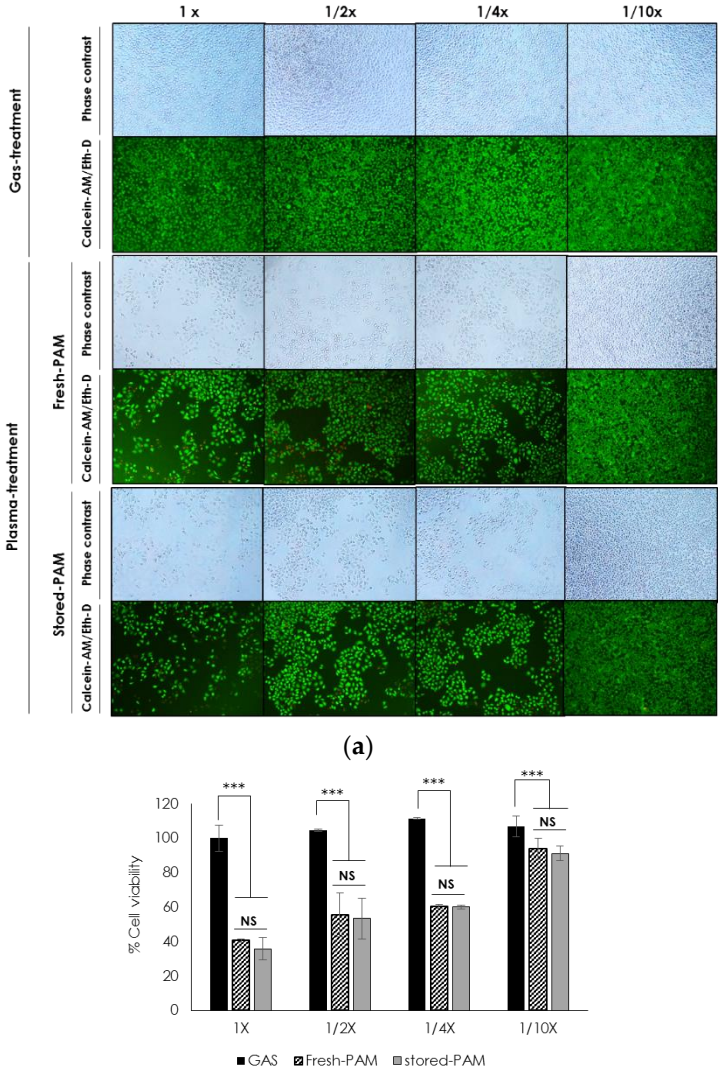
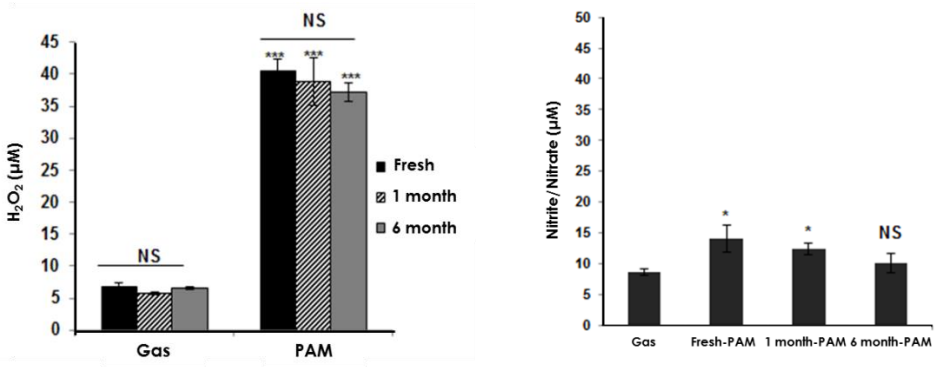


Figure 6. Quantitative assessment of anticancer efficacy of stock PAM. The fresh and 6-month stock PAM (1×) were diluted by 1/2, 1/4, and 1/10. HeLa cells were incubated in the diluted fresh and stock PAM for 24 h and then cell viability was evaluated by conducting live/dead assay (**a**) and MTT assay (**b**). NS: not significant; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.4. Maintained ROS Level in Stock Plasma Indicating the Advantage of PAM

We then monitored the levels of ROS and RNS in fresh and stock PAM (Figure 6). In agreement with the cell death levels induced by fresh and stock PAM (Figures 4–6), the levels of ROS and RNS in fresh and stock PAM were similar (Figure 7). We performed a hydrogen peroxide assay and Griess assay to analyze ROS (H_2O_2) and RNS (nitrite and nitrate), respectively. Interestingly, significantly increased levels of H_2O_2 were detected in fresh and stock PAM (37.2–40.5 μM) (Figure 7a), suggesting that the level of H_2O_2 in stock PAM was stably maintained. Modest increases in nitrite and nitrate measured by the Griess assay were observed in both fresh and stock PAM (Figure 7b) compared to in control medium, and then gradually decreased to the basal level observed in control gas-treated medium, indicating that nitrite and nitrate were sustained in the stock PAM. Taken together, these results demonstrate that PAM can be effectively stored for up to 6 months, possibly without a significant decrease in the levels of reactive ingredients.



(**a**) (**b**)

Figure 7. Reactive species in stock PAM were stably maintained. H_2O_2 (**a**) and nitrite/nitrate (**b**) in fresh and stock PAM were quantified. PAM was stored in a freezer (−20 °C) for the indicated periods (1 or 6 months) and then thawed to assess H_2O_2 and nitrite/nitrate levels. DMEM served as a control. NS, not significant; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4. Discussion

Consistent with the results of our previous study, we demonstrated that PAM had anticancer effects on HeLa cells [1,3,28]. PAM had the same effect on inducing cancer cell death as direct treatment.

The rates of cell viability or apoptosis after treatment with fresh or stock PAM were detected by live/dead, flow cytometry, or MTT assays. No large differences were observed between the fresh and stock PAM. Both plasmas induced cell death via apoptosis. This suggests that fresh and stock PAM induce cell apoptosis through the same mechanism. Our data showed that the concentration of dissolved ozone, a reactive oxygen species (ROS), increased with increased exposure time. Along with the optical emission spectrum data, the level of ROS was increased by 10-fold in treated culture media compared to non-treatment; this rate was similar in fresh and stock PAM. Additionally, RNS (nitrite and nitrate) in treated culture media showed a modest increase compared to non-treatment and a gradual decrease during stock. Consistent with previous reports [21,22,28], our findings indicate that the anticancer effect of fresh and stock PAM depend on H_2O_2 and nitrite/nitrate. Our study suggests that fresh and frozen PAM function as apoptotic inducers by generating ROS/RNS products in cellular cultured media.

Based on our results, PAM may be useful as a pharmaceutical drug. PAM shows anticancer effects equivalent to those of direct air plasma and the stock PAM showed anticancer efficacy. In summary, our results broaden the application spectrum and enhance the feasibility of plasma medicine.

Author Contributions: Authors' individual contributions are as follows: plasma generation, H.J.P.; biological experiments, N.H.N. and S.Y.H.; writing—original draft preparation, N.H.N. and H.J.P.; writing—review, J.-S.L.; writing—editing, S.S.Y.; funding acquisition, J.-S.L. and S.S.Y.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the writing of the manuscript.

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