Enhancing Cold Atmospheric Plasma Treatment of Cancer Cells by Static Magnetic Field

Xiaoqian Cheng,1 Kenan Rajjoub,2 Alexey Shashurin,3 Dayun Yan,1 Jonathan H. Sherman,4 Ka Bian,5 Ferid Murad,5 and Michael Keidar1,4*

1Department of Mechanical and Aerospace Engineering, The George Washington University, Washington, District of Columbia
2Columbian College of Arts and Sciences, The George Washington University, Washington, District of Columbia
3College of Engineering, Purdue University, West Lafayette, Indiana
4Department of Neurological Surgery, The George Washington University, Washington, District of Columbia
5Department of Biochemistry and Molecular Medicine, The George Washington University, Washington, District of Columbia

It has been reported since late 1970 that magnetic field interacts strongly with biological systems. Cold atmospheric plasma (CAP) has also been widely studied over the past few decades in physics, biology, and medicine. In this study, we propose a novel idea to combine static magnetic field (SMF) with CAP as a tool for cancer therapy. Breast cancer cells and wild type fibroblasts were cultured in 96-well plates and treated by CAP with or without SMF. Breast cancer cells MDA-MB-231 showed a significant decrease in viability after direct plasma treatment with SMF (compared to only plasma treatment). In addition, cancer cells treated by the CAP-SMF-activated medium (indirect treatment) also showed viability decrease but was slightly weaker than the direct plasma-SMF treatment. By integrating the use of SMF and CAP, we were able to discover their advantages that have yet to be utilized. Bioelectromagnetics. © 2016 Wiley Periodicals, Inc.

Keywords: cold atmospheric plasma; cancer; static magnetic field; plasma efficacy; cold plasma selectivity

INTRODUCTION

In the past few decades, cold atmospheric plasma (CAP) has been widely used in various fields such as material processing [Zelzer et al., 2012], bacterial inactivation [Deng et al., 2007], wound healing, cut coagulation [Ihsary et al., 2012], cancer therapy [Shashurin et al., 2008; Keidar et al., 2011; Barekzi and Laroussi, 2012; Walk et al., 2013], and viral destruction [Shi et al., 2012]. The temperature of heavy species in CAP is usually close to room temperature, allowing its application to living tissue treatment [Keidar, 2015].

It has been reported since the late 1970s that a magnetic field (MF) appears to have a strong effect on biological systems [Bawin and Sabbot, 1978; Liboff, 1985; Halle, 1988; Liboff and McLeod, 1988]. Research on the electromagnetic field effect on biological systems advanced after Wertheimer and Leeper [1979] found that the likelihood of developing leukemia in children increased as they were present in 60Hz frequency electromagnetic field. As the research progressed, it appeared as though vibrational energy levels in the ion–protein complex were pumping into the system, which was creating parametric resonance. This occurs when the atoms shake slightly [Lednev, 1991]. This “shaking” is an anomaly that can change ion flux through the cell membrane

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*Correspondence to: Michael Keidar, Department of Mechanical and Aerospace Engineering, School of Engineering and Applied Science, The George Washington University, Science and Engineering Hall 3550, 800 22nd Street, Northwest, Washington 20052, DC. E-mail: keidar@gwu.edu

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[Liboff et al., 1987] or cell mobility [McLeod et al., 1987; Smith et al., 1987]. Santoro et al. [1997] showed that extremely low frequency (ELF) MFs influence physiological processes in different organisms, such as plasma membrane structure modification and initiation of signal cascade pathways interference. Cell membrane morphology modification by ELF was again reaffirmed by Ikehara et al. [2003], who found that exposure to the ELF MF has reversible effects on N—H inplane bending and C—N stretching vibrations of peptide linkages, and changes the secondary structures of α-helix and β-sheet in cell membrane proteins.

Cells are also being tested in order to examine how static magnetic fields (SMFs) affect apoptosis. Based on the findings of Fanelli et al. [1999], SMFs (0.6–6 mT) exert a strong and reproducible effect of reducing U937 and CEM (normal cell lines) apoptosis. This effect is mediated by MFs’ ability to increase Ca^{2+} influx since its inhibition abrogates MFs’ antiapoptotic effect. On the other hand, Raylman et al. [1996] showed the growth of three cancerous cell lines (HTB 63, HTB 77 IP3, and CCL 86) and exhibited a significant reduction in viability after lengthy exposures (64 h) to very high uniform SMFs (7 T). Potenza et al. [2004] reported that alterations in terms of increased *Escherichia coli* cell proliferation and changes in gene expression with a long incubation time (up to 50 h) were induced by SMF.

Since the plasma has shown selectivity towards cancer therapy and studies have also demonstrated the capability of MF interaction with biological systems, we propose a novel idea that combines CAP and SMF in cancer therapy. By integrating the use of MF and CAP, we have been able to discover their advantages that have yet to be utilized.

MATERIALS AND METHODS

Cold Plasma Device

The cold plasma device created at The George Washington University (Washington, DC) has a configuration of central powered electrode of 1 mm diameter coating with 2 mm ceramic layer and a grounded outer electrode wrapped around the outside of a 4.5 mm diameter quartz tube. The electrodes were connected to a secondary high voltage resonant transformer with voltage up to 10 kV and a frequency of 30 kHz. The plasma discharge was driven by alternating current (AC) high voltage. The output voltage was set to 3.16 kV. The feeding gas helium (Airgas, Alexandria, VA) was set at a flow rate of 4.7 L/min. Distance between the CAP nozzle and the plate was set to 3.5 cm.

SMF Measurement

A permanent magnet (McMaster-Carr, Princeton, NJ) was used to provide SMF, as shown in Figure 1A. The MF strength was measured by a Gauss meter (GM08, Hirst Magnetic Instruments, Falmouth, UK). MFs at vertex, one-fourth point, center, and end on the magnet were tested (Spots A, B, C, and D shown in Fig. 1B).

Cell Culturing

Human breast cancer cells MDA-MB-231 were used in this study. In order to show the selective effect of plasma, wild type mouse dermal fibroblasts (WTDF) were also tested under the same conditions. The cells were cultured in Dulbecco’s Modified Eagle Medium (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Atlantic Biologicals, Frederick, MD) and 1% (v/v) Penicillin and Streptomycin (Life Technologies). Cultures were maintained at 37 °C in a humidified...
Cell Viability Assay

In order to compare the cell activity of plasma treatment with and without SMF, cell viability was monitored using the MTT assay (Sigma–Aldrich, St. Louis, MO), which is a colorimetric assay for measuring the activity of mitochondria and cellular dehydrogenase enzymes that reduce 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, and MTT to its insoluble formazan, giving a purple color. The detailed procedure can be found elsewhere [Cheng et al., 2014]. Briefly, cells were plated at a confluence of 30,000 ml⁻¹ and then incubated for 1 day to ensure proper cell adherence and stability. Before treatment, cells were replaced with fresh medium, and treated with direct or indirect CAP followed by additional incubation at 37 °C for 72 h. After the incubation, 100 ul of MTT solution per well (7 mg Thiazolyl Blue Tetrazolium Blue in 10 ml medium for one 96-well plate) was added into each well. Reactions were maintained for 3 h at 37 °C. The MTT solution was aspirated and 100 ul of MTT solvent (0.4% (v/v) HCl in anhydrous isopropanol) was added to each well to dissolve formazan crystals. Reactions were monitored by a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) at a wavelength of 570 nm. The entire set of experiments was repeated four times in duplicates.

Optical Emission Spectra Measurement

In this study, spectra of the plasma jet with and without the SMF presence were measured to detect the difference of reactive species variation in these two experimental conditions. The spectrometer and detection probe were purchased from Stellar Net (Tampa, FL). Integration time of the collecting data was set to 100 ms.

Intracellular ROS Measurement and Hydrogen Peroxide Measurement

For the general intracellular reactive oxygen species (ROS) measurement, 5, 6-Chloromethyl-29, 79-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was purchased from Invitrogen (Carlsbad, CA). MDA-MB-231 cells were plated in 96 well plates with 100 μl medium and treated as required. Two hours after treatment, 10 μl 10X CM-H2DCFDA solution in PBS was added in each well to reach the final concentration of 10 nM. After 30 min, the intensity of the fluorescence was read by Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments) at excitation wavelength of 492 nm and emission wavelength of 527 nm. The sensitivity of the reader was set to 100.

Hydrogen peroxide (H2O2) detection kit was purchased from Sigma–Aldrich. The experiment was performed according to the detailed protocols given on the official website. Cells were plated in black clear-bottom 96 well plates (Corning, Corning, NY). Immediately after the required treatment, the fluorescence intensity of the H2O2 was obtained with a microplate reader (Synergy H1 Hybrid Multi-Mode) at excitation wavelength of 540 nm and emission wavelength of 590 nm. Sensitivity of the reader was set to 60.

Statistics

Results of four repetitions of each experiment were plotted using Microsoft Excel software (2011) as mean ± standard deviation. Student t-test (for comparison between two groups) and one-way ANOVA (for comparison between three groups) were used to check the statistical significance (P < 0.05).

RESULTS

Cold atmospheric plasma and SMF were integrated in the way shown in Figure 1A. The location of cells on the magnet section became important because this determined the conditions of SMF such as direction and strength. Thus, the MFs at vertex,
one-fourth point, center, and end on the magnet were tested (Spots A–D illustrated in Fig. 1B). The values are shown in Table 1. The normal SMF strengths for Spots A–D are 106, 18, 1, and 1 mT, respectively; and the tangential SMF strengths for them are 26, 38, 30, and 150 mT, respectively.

Cells were plated in the 96-well plate at a density of 3000 cells per well, then treated for 30 s with plasma and MF (at A–D) after 24 h incubation. The MTT assay of cells treated by plasma with and without SMF was performed at 72 h time point after treatment, to allow the difference to magnify over incubation time. The viability of the experiments repeated three times were consistent at Spots A–C, showing a decreasing viability pattern. While at Spot D, however, the cell viability after plasma treatment demonstrated an instability (data not shown). Therefore, Spot C is the optimal treatment point that shows the most significant interaction with plasma.

The spectra of a normal helium plasma jet with or without an SMF at Spot C were measured. As shown in Figure 2, the identical spectra indicate that the SMF does not affect the generation of the reactive plasma species, such as OH (309 nm) [Winter et al., 2013], NO (296 nm) [Pipa et al., 2012], N$_2^+$ (391 nm) [Korbut et al., 2014], and O (777 nm) [Yonemori and Ono, 2014]. These species are believed to be the key species in the plasma jet to affect the biological system of the cells [Reuter et al., 2012].

To understand the interaction between SMF and plasma, cells should be isolated from the treatment. Previous studies have demonstrated that the chemical components of plasma-stimulated culture medium are modified by the plasma treatment [Takai et al., 2014], and this activated medium is also capable of inducing cancer cell death [Yan et al., 2014; Adachi et al., 2015]. The way that cells are treated by plasma-activated medium, rather than plasma directly, is termed indirect treatment in the rest of the text and figures, while cells treated by plasma jet are direct treatment. The indirect treatment offers the possibility of cell isolation from the system of SMF and plasma.

Cell viability assay was performed to understand the interaction between SMF, plasma, and cells. All the cells were also plated at a density of 3000/well and incubated for 24 h before treatment. The MTT assay was performed at 72 h time point after treatment. We accessed the viability of cancerous and normal cell lines directly and indirectly treated by helium plasma jet only, plasma jet with an SMF, and plasma jet with a non-magnetic ferrite bar at Spot C.

CAP jet, a weakly ionized gas, can be intensified by coupling it to the conductive plate. Therefore, with magnet itself being a conductor, the distance between the nozzle of the plasma device and magnet surface is delicate. If the distance is too far, the intensity of the plasma jet will be significantly reduced so that very little amount of reactive species can reach the medium or cells; while if it is too close, the CAP jet will be enhanced at the tip of the jet where it is in contact with the medium. The plasma jet coupled to conducting plate can lead to a much higher amount of the ionized species than the plasma treatment without a magnet, making the data incomparable. Therefore, a non-magnetic ferrite bar (NMF) of the same material was used as a conductor to replace the magnet. NMF can eliminate the factor of the plasma jet enhancement. Results are shown in Figure 3. The cell viability was normalized to plasma treatment only.

**TABLE 1. MF Strength of Different Locations on the Permanent Magnet**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Normal SMF strength (mT)</th>
<th>Tangential SMF strength (mT)</th>
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<tbody>
<tr>
<td>A</td>
<td>106</td>
<td>26</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>150</td>
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*Fig. 2. Spectra of helium plasma jet (top) and plasma jet with SMF under jet (bottom).*
A one-way analysis of variance (ANOVA) test was performed between the cell viability by direct treatment of CAP, CAP with SMF, and CAP with NMF (left half of Fig. 3A). The \( P \)-value of this ANOVA test is 1.798E-11, indicating that there are statistical differences between the three treatments. Thus Student \( t \)-test was performed between the groups of CAP and “CAP + SMF,” CAP and “CAP + NMF” to determine where the significance lay. As presented in Figure 3A, viability of the cells treated directly by CAP is close to the ones treated by CAP with NMF (\( P \)-value of the \( t \)-test is 0.169, no statistically significant difference). The \( P \)-value of the \( t \)-test between CAP and “CAP + SMF” is 2.84E-07, which confirms the significance. The same calculation was also performed on the indirect treatments (Fig. 3B). The \( P \)-value of CAP and “CAP + SMF” is 7.086E-06, suggesting that the significance also exists in indirect treatment. Figure 3C shows the viability of cells treated by plasma with SMF compared to only plasma treatment in direct and indirect ways. The SMF induced 25% more cell death when cells were treated by plasma directly, and 20% more cell death when cells were treated indirectly. The \( P \)-value of direct and indirect treatment is 0.0379, suggesting a statistically significant difference.

In order to prove that the SMF effect alone does not have the ability to activate the medium or induce cell death, the cell culture medium and cells were placed in the SMF for 30 s, respectively. The SMF-treated medium was then transferred to infect the pre-plated cells immediately (indirect treatment). Untreated MDA-MB-231 cells were used as a negative control. Cell viability obtained after 72 h of incubation is shown in Figure 4A. The viability of neither SMF-directly treated cells nor cells treated by SMF-activated medium is significantly different from the viability of the untreated cells (\( P \)-value of ANOVA test is 0.5127), proving that...
effect of the 30 s SMF exposure alone can be ruled out from investigation of the mechanism. Figure 4B is the MTT results of cells treated by CAP and SMF directly (denoted as P + SMF), and cells pre-incubated in the SMF for 1 h, then treated with plasma and SMF directly (denoted as CAP + 1 h + SMF). Cell viability was also normalized to the untreated group. Although the P-value of t-test is 0.0944, meaning the cell viability of 1 h pre-incubated cells is not significantly different from cells treated by CAP in SMF, the descending trend is consistent (repeated three times). A longer pre-incubation might cause a significant viability decrease. This is beyond the scope of this study, thus is not reported here.

To further illustrate that SMF does not change the CAP configuration, the generation of H₂O₂ in the culture medium treated by CAP and CAP with SMF was measured (data were normalized to CAP treatment). In Figure 5, similar H₂O₂ level was found in the medium treated by CAP, CAP with MF. The P-value of t-test is 0.199, indicating that the CAP configuration remains consistent with the application of the SMF.

The CAP treatment on cells can lead to an increased level of free radicals, which has an impact on cellular activity and explains the decrease of cell viability. Therefore, to determine if ROS pathways involved in the mechanism of CAP together with SMF further decrease cell viability, the production of intracellular ROS were assessed in cells treated by CAP, and CAP with SMF. As shown in Figure 6, no significant difference in the ROS intensity was observed (P-value of t-test is 0.0684), suggesting that ROS pathways are not the dominant mechanism of this study. However, the P-value is very close to 0.05, which could imply that ROS did have a role in this reaction, but this effect was diminished by other factors which are yet unknown.

Wild type mouse dermal fibroblasts (WTDF) shows no significant difference between the treatment of CAP and CAP + SMF both directly or indirectly (Fig. 7). Results of WTDF indicate that plasma treatment leads to the decrease of viability of normal cells around 15%, while the decrease of breast cancer cells MDA-MB-231 is around 60% (Fig. 3A). Statistically speaking, the odds of cancer cells killed by plasma treatment are 60:40, or 6:4 = 1.5:1, while the odds of normal cells killed by plasma treatment are 15:85, or 15:85 = 0.176:1. Thus the odds ratio is 1.5:0.176 = 8.5, which means the MDA-MB-231 cells have 8.5 times the odds than WTDF to have been killed by plasma, confirming the selectivity of plasma treatment that we have previously studied on other cancerous and normal cell lines [Keidar et al., 2011; Cheng et al., 2014].

**DISCUSSION**

The data presented in this study show that plasma alone, and in combination with SMF, can
selectively induce cancerous cell death. To understand the possible mechanism influence of SMF on plasma treatment in cells, the interaction between two of the three parties should be primarily figured out. Thus, we will individually discuss the interaction between plasma and cells, plasma and SMF, and SMF and cells.

The interaction between cells and plasma has been intensively investigated [Ahn et al., 2011; Arjunan et al., 2012; Brulle et al., 2012; Iseki et al., 2012; Arndt et al., 2013]. In terms of mechanism of cancer therapy, the majority favors the theory of reactive oxygen and nitrogen species (ROS and RNS) generated by plasma (extracellular ROS/RNS) and the intracellular ROS signaling and apoptotic pathways they induce. The intracellular ROS generation is promoted by plasma, which could cause cell death by impairing the function of intracellular regulatory factors [Ishaq et al., 2014; Ma et al., 2014]. Recent studies have emphasized the importance of H$_2$O$_2$ formation in the culture medium treated by plasma. Liu et al. [2010] suggested that H$_2$O$_2$ is majorly formed by 2 *OH radicals. Bekeschus et al. [2015] varied the shielding gas, which affects the generation of *OH in the plasma jet in order to control the H$_2$O$_2$. They found that the toxicity of plasma is highly dependent on H$_2$O$_2$, which has a dominant role in the mechanism of cell death. On the other hand, RNS, especially NO and peroxynitrite (ONOOH), are also considered important species that lead to cell death [Virag et al., 2003; Pacher et al., 2007]. Lukes et al. [2014] show that peroxynitrite formation in the plasma activated medium is through the reaction of NO$_3$ with H$_2$O$_2$ and H$^+$. ONOOH is a powerful oxidant and nitrating agent that is known to be much more damaging to cells than NO or superoxide because cells readily remove superoxide and NO to reduce their harmful effects, while fail to neutralize ONOOH.

In the system of plasma and SMF, it is clear from the spectra that the addition of the external SMF does not alter the plasma chemical composition. H$_2$O$_2$ production measurement in the medium further confirms the stability. The consistency of plasma jet composition guarantees that effects observed here are associated with SMF interaction with cells and reactive species in the medium. The $P$-value of the production of intracellular ROS assessed in cells treated by CAP and CAP with SMF was 0.0684. On one hand, it is greater than 0.05, suggesting that ROS pathways might not be the dominant mechanism of this study. However, with $P$-value close to 0.05, it must be acknowledged that the role of ROS could be diminished by the combination effect of SMF, CAP, and cells.

The consumption of H$_2$O$_2$ by cells over time has been studied by Yan et al. [2015]. They demonstrated that each cell line consumed H$_2$O$_2$ at different rates. Adachi et al. [2015] presented that the concentration of H$_2$O$_2$ halved when stored at room temperature in comparison to $-80^\circ$C after 3 days. However, the H$_2$O$_2$ decay at different time intervals within 1 day (0, 30 min, 1, 6, and 24 h) has a lack of investigation. Possible future experiments could help discover more about the role of ROS by treating medium with CAP and CAP + SMF then adding it to the cells after different time intervals from 0 to 3 days. Cell viability of each post-delayed addition of medium can be measured to support the decay of H$_2$O$_2$.

To further confirm that the SMF interacted with the biological system of cells rather than the plasma jet, the culture medium activated by normal plasma jet as well as the jet with SMF was used to affect the cells prepared in the 96-well plates. This indirect treatment will isolate cells from the system of SMF and plasma, allowing us to understand if the interaction is solely between the SMF and cells. The results of indirect treatment experiments above have shown that the CAP-SMF-activated medium can also increase the cell death rate compared to the CAP-activated medium. However, the cell death rate of indirect treatment is statistically significant, but lower than that of direct treatment, suggesting that the mechanism of cells killed by plasma in SMF could be an outcome of two separate reactions: SMF with cells and SMF with the CAP-activated medium.

As for MF interaction with cells, previous studies have shown both static [Raylman et al., 1996; Fanelli et al., 1999; Potenza et al., 2004] and ELF [Ikehara et al., 2003; Ledda et al., 2013] MFs can interact with biological systems. While others have demonstrated that prolonged exposure in the SMF can inhibit human cancer cell growth [Raylman et al., 1996] and increase normal cell survival [Fanelli et al., 1999], in our case, 30 s SMF treatment alone does not induce cell death. Thus the role of SMF effect on the cells can be ruled out of the exploration of mechanism because the cells were not incubated with SMF for a long period of time as the above studies did.

Cancer cells and normal cells differ in their cell–cell communication, characteristic cell death, repair mechanisms, or other cellular activities [Shaw et al., 2014]. As normal cells, WTDI are included in this study to demonstrate the selectivity of plasma treatment. Breast cancer cells have 8.5 times higher odds to be killed by plasma treatment than WTDI. To
make sure plasma and SMF treatment only affect the viability of cancer cells, plasma can be assembled with an endoscope, targeting only the tumor area. The other way to bypass the effect of SMF on normal cells is to use magnetic nanoparticles, which can target only cancer cells when guided by a magnet or conjugated with a targeting antibody. Both techniques as supplementary experiments to this study are currently under research.

Finally, in the system of cells, plasma, and SMF, as discussed above, plasma will generate extracellular ROS and RNS species in medium, such as *OH, H$_2$O$_2$, O$_2^-$, NO$_2^-$, NO$_3^-$, and ONOOH. Plasma-produced ROS (or their reaction products) in medium can either diffuse through the plasma membrane or react with the plasma membrane to produce intracellular ROS. Once ROS enter cells, they can damage intracellular components, promoting or inhibiting intracellular signaling pathways. Therefore, a possible way to explore the mechanism of CAP-SMF synergy is to monitor intracellular ROS production. However, the matching ROS level in the cells treated by CAP and CAP with SMF indicates that ROS might not be the dominating mechanism in the system of cells, CAP, and SMF.

Although there is no consensus regarding the mechanism of the interaction between MF and biological systems given in the literature, this paper is to report a novel idea of improving cold plasma effect on cancer cells. While this paper does not reveal the exact mechanism of how the combination of SMF and CAP affects cellular pathways, it does prove that the SMF stimulates ions in biological systems and reactive species in the plasma treated medium, providing a more efficient way for cancer therapy.

CONCLUSIONS

As proven by numerous studies, cold atmospheric plasma can kill various kinds of cancer but how to enhance the efficiency of cold atmospheric plasma on cancer therapy has barely been studied. Although plasma can selectively kill cancer cells, long-time exposure can still damage normal cells around the tumor. This prompts researchers to seek novel ideas in the designing of plasma treatment. This study provides the idea of combining cold atmospheric plasma and SMF in order to achieve the enhanced killing effect on cancer cells. In the system of plasma, cells, and SMF, SMF enhances the efficiency of plasma on cancer therapy through the way of interfering with the cell biological system and reactive species instead of interacting with plasma jet. In addition, SMF can be used to guide plasma–cell interaction region. As such it has promise to enhance selectivity.

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