Treatment of gastric cancer cells with non-thermal atmospheric plasma generated in water

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Abstract

Non-thermal atmospheric plasma (NTAP) can be applied to living tissues and cells as a novel technology for cancer therapy. Even though studies report on the successful use of NTAP to directly irradiate cancer cells, this technology can cause cell death only in the upper 3-5 cell layers. We report on a NTAP argon solution generated in DI water for treating human gastric cancer cells (NCI-N87). Our findings showed that the plasma generated in DI water during a 30-minute treatment had the strongest affect in inducing apoptosis in cultured human gastric cancer cells. This result can be attributed to presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced in water during treatment. Furthermore, the data showed that elevated levels of RNS may play an even more significant role than ROS in the rate of apoptosis in gastric cancer cells.

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Introduction

Gastric cancer is one of the most aggressive types of carcinomas and it is known as the second most common cause of death\textsuperscript{1,2}. Worldwide, the number of newly diagnosed cases is predicted to reach 930,000 per year, while China alone accounts for 42\% of them\textsuperscript{3}. Although gastric cancer is usually managed by chemotherapy or surgery, its 5-year survival rate is approximately 15\%\textsuperscript{4}. Therefore, efforts to improve survival rates of patients with gastric cancer are one of the main challenges in current research.

Non-thermal atmospheric plasma (NTAP) can be applied to living cells and tissues due to selective cell death without influencing the healthy tissue\textsuperscript{5-9}. The unique properties of NTAP have enabled recent biomedical applications including wood healing\textsuperscript{10}, sterilization\textsuperscript{11}, blood coagulation\textsuperscript{12}, tooth bleaching\textsuperscript{13}, skin regeneration\textsuperscript{14} and cancer therapy\textsuperscript{15-17}. NTAP is known for the generation of charge particles, electronically excited atoms, ROS, RNS, etc\textsuperscript{18}. ROS and RNS, combined or independently, are known to induce cell proliferation as well as cell death. Additionally, extreme amounts of reactive species may lead to the damage of DNA, proteins, lipids, senescence and induce apoptosis\textsuperscript{19,20}. Recent studies showed that indirect NTAP therapy can significantly affect cancer cells\textsuperscript{21}. However, there are no studies that report on using NTAP for treating gastric cancer cells, let alone that use NTAP generated in DI water.

This paper presents the effects of plasma in gastric cancer cells. The plasma solution was obtained using NTAP generated in DI water. This study describes the NTAP device, its characterization and the response of cancer cells to the plasma solution therapy. The voltage and current of NTAP generated in DI water were measured with a Tektronix TDS 2024B Oscilloscope. The spectra of NTAP generated in DI water were characterized by UV-visible-NIR Optical Emission Spectroscopy. The plasma density was monitored by Rayleigh Microwave Scattering system
(RMS). The temperature of plasma solution was measured with FLIR Systems Thermal Imaging.

The concentrations of ROS and RNS in DI water were determined by using a Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, MO), and the Griess Reagent System (Promega, WI). The cell viability of the human gastric cancer cell line (NCl-N87) was monitored with the Cell Counting Kit 8 assay (Dojindo Molecular Technologies, MD).

The utilized plasma device is shown in Fig. 1a. Industrial grade argon with a flow rate of about 0.4 L/min was used for testing. The device consisted of 2 electrodes submerged in water. One electrode was a central powered electrode (1 mm in diameter) and the other one was a grounded outer electrode wrapped around the outside of a quartz tube (4.5 mm in diameter). Deionized water was treated with CAP generated in water for 5, 10, 20, and 30 mins, respectively. The two electrodes were connected with a high voltage power supply. The graphs of CAP for current and voltage are shown in Fig. 1b, where the peak-peak voltage is about 8 kV and the average current is 0.23 mA. The frequency of the discharge generated in DI water is around 6.25 kHz. The temperature change of the plasma solutions for different treatment durations is shown in Fig. 1c. The graph shows that the temperature rises with increased treatment duration. The highest temperature increase of 34.4 ± 1.6 °C is achieved at 30 minutes’ plasma treatment. The reactive species that are produced by CAP generated in DI water are shown in Fig. 1d. The identification of emission lines and bands was performed according to the reference22. High-intensity OH/O₃ peak at 309 nm and low-intensity N₂ second-positive system (C³Πᵤ – B³Π₉) with its peaks at 337, 358, and 381 nm were observed. Naturally argon lines observed in the range of 600 and 800 nm are shown in Fig. 1d.
Fig. 1. The Non-thermal atmospheric plasma (NTAP) generated in DI water. (a) Schematic diagram of NTAP device setup consists of a HV pulse generator connected to a pin-to-plate electrode system submerged in DI water. (b) Voltage and current graph for CAP device. (C) Temperature changes of plasma solution with treating time increasing. (d) Optical emission spectrum detected from the plasma submerged in DI water using UV-visible-NIR, a range of wavelength 250-850 nm.

The experimental Rayleigh Microwave Scattering System (RMS) are presented in Fig. 2a. Microwave radiation linearly polarized was scattered on the collinearly-oriented plasma channel and then the scattered signal was measured. Two microwave horns were used for radiation and detection of the microwave signal. A homodyne I/Q Mixer providing in-phase (I) and quadrature (Q) outputs was used to detect the scattered signal. For the entire range of scattered signals, the amplifiers and mixer were operated in linear mode. The total amplitude of the scattered microwave
signal was determined by: \( U = \sqrt{I^2 + Q^2} \). The dependence of the output RMS signal on parameters of the scattering channel can be expressed as \( U = A\sigma V \), where \( A \) is the proportionality coefficient (\( A = 263.8 \, V \Omega/cm^2 \)) and \( V \) is the plasma volume. The volume of the plasma column was determined from the Intensified Charged-Coupled Device (ICCD) images. \( \sigma \) is plasma conductivity by using the following expression: \( \sigma = 2.82 \times 10^{-4} n_e v_m / (w^2 + v_m^2) \), \( \Omega^{-1} cm^{-1} \), where \( v_m \) is the frequency of the electron-neutral collisions, \( n_e \) is the plasma density, and \( w \) is the angular frequency. Temporal evolution of argon plasma density is presented in Fig. 2b and its average electron density was \( 2.55 \times 10^{13} \, cm^{-3} \).

![Diagram of experimental setup](image.png)

**Fig. 2.** The schematics of RMS experimental setup (a) and temporal evolution of plasma density in Ar non-thermal atmospheric plasma jet (b)
NTAP can produce chemically active species in DI water. Plasma discharge produced ROS and RNS in DI water in a time-dependent manner, as shown in Fig. 3a and Fig. 3b, respectively. In order to compare generation efficiency of ROS and RNS, we measured concentration of H₂O₂ and NO₂⁻ in the case of the submerged NTAP device. H₂O₂ was produced in DI water within a few microseconds from hydroxyl (•OH)²⁴. On the other hand, gas phase H₂O₂ in the afterglow also solvates into the DI water. Following mechanisms of H₂O₂ formation in our cases can be suggested²⁰,²⁵-²⁸.

\[
\text{Ar} \rightarrow \text{Ar}^+ + e \tag{1} \\
\text{Ar}^+ + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + \text{Ar} \tag{2} \\
\text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \cdot\text{OH} \tag{3} \\
\text{Ar} + e \rightarrow \text{Ar}^* + e \tag{5} \\
\text{Ar}^* + \text{H}_2\text{O} \rightarrow \text{Ar} + \cdot\text{OH} + \text{H}^* \tag{6} \\
e + \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^* + e \tag{7}
\]
\[
\begin{align*}
\text{H}_2\text{O}^* & \rightarrow \text{•OH} + \text{H•} & (8) \\
hv + \text{H}_2\text{O} & \rightarrow \text{•OH} + \text{H•} & (9) \\
\text{•OH} + \text{•OH} & \rightarrow \text{H}_2\text{O}_2 & (10)
\end{align*}
\]

According to Arrhenius theory\textsuperscript{29}, the decomposition rate of \( \text{H}_2\text{O}_2 \) increases with temperature. The temperature of the plasma solution increased with treatment time (Fig.1c), which might explain the decrease in \( \text{H}_2\text{O}_2 \) concentration.

Fig. 3b shows that the \( \text{NO}_2^- \) concentration increases with treatment time. The \( \text{NO}_2^- \) mainly originates as \( \text{NO} \), while most of \( \text{NO} \) is formed in the gas phase during the afterglow a few milliseconds after the discharge pulse. It is known that \( \text{NO}_2^- \) is a primary breakdown product of \( \text{NO} \) in DI water\textsuperscript{30} and through the following pathway\textsuperscript{31}.

\[
\begin{align*}
\text{N}_2 + \text{e} & \rightarrow 2\text{N} + \text{e} & (11) \\
\text{N} + \text{O}_2 & \rightarrow \text{NO} + \text{O} & (12) \\
4\text{NO} + \text{O}_2 + 2\text{H}_2\text{O} & \rightarrow 4\text{NO}_2^- + 4\text{H}^+ & (13)
\end{align*}
\]

Due to DI water contact with air, it is plausible to assume that \( \text{O}_2 \) and perhaps \( \text{N}_2 \) is coming from air. On the other hand, \( \text{N}_2 \) is perhaps coming from the industrial grade argon. Thus reactions (11), (12) and (13) can be used to explain the production \( \text{NO}_2^- \) in argon gas
Plasma generated in DI water was applied to gastric cancer cells. DMEM and untreated DI water was used as the control conditions. Fig. 4 shows the viability of the human gastric cancer when they were exposed to DMEM, DI water and plasma solution (5, 10, 20, and 30 mins) for 24 hours and 48 hours. At 24 hours, the viability decreased by 15.8% when the cells were treated with DI water in comparison with the DMEM control condition (Fig. 4a). The viability of cells treated by plasma solution was lower than that of the DI water and decreased with increasing treatment time. At 48 hours the viability of the cell decreased by approximately 22.6%, 28.2%, 30.9%, 41.6%, and 45.7% respectively according to treatment duration. (Fig. 4(b)). A decrease in cell viability was accompanied with an increase in the concentration of nitrite and a decrease in the concentration of H$_2$O$_2$. Thus, it can be seen that the strongest effect can be observed at 30 min plasma solution.

ROS and RNS are important signal mediators that regulate cell death$^{30}$. When the cell is stimulated by environmental stress or other factors, it produces ROS that are potential signaling molecules$^{32}$. Extreme amount of ROS in the cells may cause DNA damage, genetic instability, cellular injury and eventually induce apoptosis. RNS is a pleiotropic mediator and a signaling molecule involved
in a large number of cell functions\textsuperscript{33}. In some situations, RNS activate the transduction pathways causing cells apoptosis and are capable of inducing cell death via DNA double-strands break/apoptosis\textsuperscript{34,35}. Our results in Fig. 3 show that the ROS concentration is highest at 5-minute treatment while the RNS concentration is highest at 30 minutes. The trend of cell death can be attributed to the increase of RNS concentration with increasing treatment time. A synergistic effect of RNS and ROS is suspected to play a key role in the apoptosis effect of plasma solution. In fact, RNS plays a more important role that ROS in gastric cancer cell apoptosis under the present experimental condition.

In summary, non-thermal atmospheric plasma was generated in DI water using argon as a carrier gas. ROS concentration decreased with extended treatment time, while RNS concentration increased with treatment. It can also be concluded that RNS plays a more significant role in gastric cancer cells death or apoptosis than ROS.

**Acknowledgement.** This work was supported in part by a National Science Foundation, grant 1465061. The authors acknowledge Dr. Alexey Shashurin from the School of Aeronautics and Astronautics, Purdue University for his assistance in setting up the RMS experiment and useful discussions. We thank Dr. Ka Bian and Dr. Ferid Murad from Department of Biochemistry and Molecular Medicine at The George Washington University for their help with the ROS and RNS experiments.
References

1 Bang, Y.-J. et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *The Lancet* **376**, 687-697 (2010).

2 Kamangar, F., Dores, G. M. & Anderson, W. F. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *Journal of clinical oncology* **24**, 2137-2150 (2006).

3 Parkin, D. M., Bray, F., Ferlay, J. & Pisani, P. Global cancer statistics, 2002. *CA: a cancer journal for clinicians* **55**, 74-108 (2005).

4 Dhar, D. K. et al. Prognosis of T4 gastric carcinoma patients: an appraisal of aggressive surgical treatment. *Journal of surgical oncology* **76**, 278-282 (2001).

5 Keidar, M. et al. Cold atmospheric plasma in cancer therapy. *Physics of Plasmas (1994-present)* **20**, 057101 (2013).

6 Keidar, M. Plasma for cancer treatment. *Plasma Sources Science and Technology* **24**, 033001 (2015).

7 Keidar, M. et al. Cold plasma selectivity and the possibility of a paradigm shift in cancer therapy. *British journal of cancer* **105**, 1295-1301 (2011).

8 Volotskova, O., Hawley, T. S., Stepp, M. A. & Keidar, M. Targeting the cancer cell cycle by cold atmospheric plasma. *Scientific reports* **2** (2012).

9 Ratovitski, E. A. et al. Anti-cancer therapies of 21st century: novel approach to treat human cancers using cold atmospheric plasma. *Plasma Processes and Polymers* **11**, 1128-1137 (2014).

10 Arndt, S. et al. Cold atmospheric plasma (CAP) changes gene expression of key molecules of the wound healing machinery and improves wound healing in vitro and in vivo. *PloS one* **8**, e79325 (2013).

11 Lee, K., Paek, K.-h., Ju, W. & Lee, Y. Sterilization of bacteria, yeast, and bacterial endospores by atmospheric-pressure cold plasma using helium and oxygen. *JOURNAL OF MICROBIOLOGY-SEOUL* **44**, 269 (2006).

12 Kalghatgi, S. U. et al. Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma. *Plasma Science, IEEE Transactions on* **35**, 1559-1566 (2007).

13 Pan, J. et al. A novel method of tooth whitening using cold plasma microjet driven by direct current in atmospheric-pressure air. *Plasma Science, IEEE Transactions on* **38**, 3143-3151 (2010).

14 Bogle, M. A., Arndt, K. A. & Dover, J. S. Evaluation of plasma skin regeneration technology in low-energy full-facial rejuvenation. *Archives of dermatology* **143**, 168-174 (2007).

15 Cheng, X. et al. The effect of tuning cold plasma composition on glioblastoma cell viability. *PloS one* **9**, e98652 (2014).

16 Cheng, X. et al. Synergistic effect of gold nanoparticles and cold plasma on glioblastoma cancer therapy. *Journal of Physics D: Applied Physics* **47**, 335402 (2014).

17 Cheng, X. et al. Cold Plasma Accelerates the Uptake of Gold Nanoparticles Into Glioblastoma Cells. *Plasma Processes and Polymers* **12**, 1364-1369 (2015).
Kumar, N. et al. Induced apoptosis in melanocytes cancer cell and oxidation in biomolecules through deuterium oxide generated from atmospheric pressure non-thermal plasma jet. *Scientific reports* **4** (2014).

Attri, P., Arora, B. & Choi, E. H. Utility of plasma: a new road from physics to chemistry. *RSC Advances* **3**, 12540-12567 (2013).

Attri, P. *et al.* Influence of ionic liquid and ionic salt on protein against the reactive species generated using dielectric barrier discharge plasma. *Scientific reports* **5** (2015).

Adachi, T. *et al.* Plasma-activated medium induces A549 cell injury via a spiral apoptotic cascade involving the mitochondrial–nuclear network. *Free Radical Biology and Medicine* **79**, 28-44 (2015).

Pearse, R. W. B., Gaydon, A. G., Pearse, R. W. B. & Gaydon, A. G. *The identification of molecular spectra.* Vol. 297 (Chapman and Hall London, 1976).

Shashurin, A., Shneider, M., Dogariu, A., Miles, R. & Keidar, M. Temporary-resolved measurement of electron density in small atmospheric plasmas. *Applied Physics Letters* **96**, 171502 (2010).

Tian, W. & Kushner, M. J. Atmospheric pressure dielectric barrier discharges interacting with liquid covered tissue. *Journal of Physics D: Applied Physics* **47**, 165201 (2014).

Van Gils, C., Hofmann, S., Boekema, B., Brandenburg, R. & Bruggeman, P. Mechanisms of bacterial inactivation in the liquid phase induced by a remote RF cold atmospheric pressure plasma jet. *Journal of Physics D: Applied Physics* **46**, 175203 (2013).

Locke, B. R. & Thagard, S. M. Analysis and review of chemical reactions and transport processes in pulsed electrical discharge plasma formed directly in liquid water. *Plasma Chemistry and Plasma Processing* **32**, 875-917 (2012).

Locke, B. R. & Shih, K.-Y. Review of the methods to form hydrogen peroxide in electrical discharge plasma with liquid water. *Plasma Sources Science and Technology* **20**, 034006 (2011).

Chen, Z., Cheng, X., Lin, L. & Keidar, M. Cold Atmospheric Plasma Generated in Water and its Potential Use in Cancer Therapy. *arXiv preprint arXiv:1604.03051* (2016).

Yazici, E. & Deveci, H. in *Proceedings of the XIIth International Mineral Processing Symposium*. 609-616.

Yan, X. *et al.* Plasma-Induced Death of HepG2 Cancer Cells: Intracellular Effects of Reactive Species. *Plasma Processes and Polymers* **9**, 59-66 (2012).

Popov, N. Associative ionization reactions involving excited atoms in nitrogen plasma. *Plasma physics reports* **35**, 436-449 (2009).

Zhang, G.-G. *et al.* Involvement of the endothelial DDAH/ADMA pathway in nitroglycerin tolerance: the role of ALDH2. *Life sciences* **82**, 699-707 (2008).

Kolb, J. Mechanisms involved in the pro-and anti-apoptotic role of NO in human leukemia. *Leukemia* **14**, 1685-1694 (2000).

Partecke, L. I. *et al.* Tissue tolerable plasma (TTP) induces apoptosis in pancreatic cancer cells in vitro and in vivo. *BMC cancer* **12**, 473 (2012).

Koo, H.-N. *et al.* Inulin stimulates NO synthesis via activation of PKC-α and protein tyrosine kinase, resulting in the activation of NF-κB by IFN-γ-primed RAW 264.7 cells. *The Journal of nutritional biochemistry* **14**, 598-605 (2003).