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Precision Tuning of a kHz-Driven Argon Plasma Jet Enables Dose-Controlled H_2O_2 Delivery to Overcome Chemoresistance in Colorectal Cancer

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Abstract

Colorectal cancer presents a significant therapeutic challenge, largely due to robust chemoresistance mechanisms, including the upregulation of antioxidant pathways. While cold atmospheric plasma is a promising anti-cancer modality, its efficacy can be limited by these cellular defenses. This study introduces a kilohertz AC-driven argon plasma jet with independently tunable voltage (1–20 kV) and frequency (18–28 kHz) as a novel platform for overcoming this resistance. We demonstrate that precision tuning of these electrical parameters allows for the controlled delivery of extracellular hydrogen peroxide (H_2O_2), a key long-lived reactive species. In the chemoresistant HT29 colorectal cancer cell line, we achieved a modulation of H_2O_2 concentrations in the culture medium, ranging from 291 to 371 µM. This H_2O_2 dosage showed a linear correlation with dose-dependent cytotoxicity ($R^2 = 0.995$, $p < 0.001$). Optimized parameters (10.5 kV, 28 kHz) overwhelmed the cells' redox defenses, reducing viability to 9.2% ± 3.6% after a 3-minute treatment. This approach successfully bypasses the Nrf2/Srx antioxidant pathway, which is known to confer resistance to helium plasma jets. Our findings establish that precisely controlling H_2O_2 delivery via a tunable argon plasma jet is a potent strategy for circumventing intrinsic chemoresistance in colorectal cancer, positioning this technology as a promising modality for precision oncology.

Keywords: Argon plasma jet; Colorectal cancer; Chemoresistance; Hydrogen peroxide (H_2O_2).

Introduction

Colorectal cancer (CRC) remains a leading cause of cancer-related mortality worldwide, with projections indicating a growing global burden (1, 2). Clinical outcomes are often compromised by high recurrence rates, driven by intrinsic therapeutic resistance and the molecular heterogeneity of tumors (3, 4). This persistent challenge underscores the urgent need for innovative therapeutic strategies capable of overcoming cellular resistance mechanisms to improve patient outcomes (5).

Cold atmospheric plasma (CAP), a partially ionized gas generated at near-body temperature, has emerged as a promising modality in oncology (6-9). CAP produces a complex cocktail of reactive oxygen and nitrogen species (RONS), including both short-lived radicals (e.g., •OH,

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$^1O_2$) and long-lived molecules (e.g., H_2O_2, NO_2^-) (10). The therapeutic principle relies on inducing overwhelming oxidative stress in cancer cells, which often exhibit a dysregulated redox homeostasis, thereby triggering apoptosis (11). Among these species, hydrogen peroxide (H_2O_2) is recognized as a key long-lived effector molecule in CAP-mediated anticancer activity, capable of diffusing through tissues and initiating sustained cytotoxic signaling (12).

The biological effect of CAP is not uniform; it is critically dependent on the device configuration (e.g., dielectric barrier discharge (DBD) vs. plasma jet) and operating parameters, which dictate the composition and flux of the generated RONS (13-16). However, a significant hurdle for CAP therapy is the adaptive response of chemoresistant cancer cells. The HT29 CRC cell line, for example, is notoriously resistant to oxidative stress due to its upregulation of potent antioxidant defense pathways, particularly the Nrf2/Sulfiredoxin (Srx) axis (17). A seminal study by Ishaq et al. (2014) demonstrated that this robust antioxidant system allowed HT29 cells to effectively neutralize RONS generated by a helium plasma jet, thus mitigating its therapeutic effect (17). This finding underscores the critical need for a CAP system capable of delivering a RONS dosage potent and controlled enough to overwhelm these protective cellular mechanisms.

To contextualize our approach, it is crucial to review prior studies investigating CAP's efficacy on the HT29 cell line, which are summarized in Table 1. The initial pivotal study by Ishaq et al. utilized a helium-based plasma jet and revealed that HT29 cells exhibit significant resistance due to the upregulation of the Nrf2/Srx antioxidant pathway (17). This resistance was only overcome when the protective pathway was silenced via siRNA, highlighting the need for a more potent CAP source. Subsequent research predominantly employed DBDs operating with ambient air. These studies, often conducted by researchers in the biological sciences, successfully induced apoptosis in HT29 cells without genetic intervention by exploring various molecular pathways, including p53-independent mechanisms and cell cycle arrest (18-21). For instance, studies utilized commercial devices like the Piezobrush® PZ2 and miniFlatPlaSter, which were originally designed for industrial surface processing or other medical applications and operate with fixed parameters (19, 20). Most recently, Martinet et al. investigated a commercial argon-based electrosurgical device (APC 3 VIO 3), originally intended for hemostasis (blood coagulation), and demonstrated its cytotoxic potential in a "cold" operating mode (22). While these studies confirmed the general anti-cancer potential of CAP, they largely relied on devices with fixed or limitedly adjustable operating parameters. This "one-size-fits-all" approach does not account for the specific redox threshold of highly resistant cells like HT29. This underscores a critical limitation: the lack of a systematic approach to tune plasma chemistry to specifically overwhelm known resistance mechanisms.

Our work directly addresses this limitation by introducing a fully tunable kHz AC-driven argon plasma jet engineered for this precise purpose. We hypothesized that the precise control afforded by this system would enable a targeted and dose-controlled delivery of H_2O_2 sufficient to surpass the antioxidant threshold of resistant HT29 cells. Unlike fixed-parameter systems (such as radio-frequency (RF) jets) or less targetable DBDs, our approach allows for the systematic optimization of plasma chemistry to maximize cytotoxicity. This study

Table 1. Summary of previous studies investigating the effects of CAP on the HT29 colorectal cancer cell line.

Study (year)	CAP Source/ Device	Working Gas	Key Findings	Ref.
Ishaq (2014)	Plasma jet	Helium	HT29 cells are resistant via the Nrf2/Srx pathway; cytotoxicity requires siRNA silencing of this pathway.	(17)
Han (2017)	DBD	Air	Induces cell cycle arrest and apoptosis via regulation of Sp1 transcription factor.	(21)
Schneider (2018)	DBD/miniFlatPlaSter	Air	Inhibits cancer cell growth regardless of p53 mutation status; non-toxic to normal colon tissue	(20)
Wang (2022)	DBD/Piezobrush® PZ2	Air	Induces mitochondrial apoptosis pathway (cytochrome c, caspase-9/3 activation).	(19)
He (2024)	DBD	Air	Induces apoptosis via the cytochrome c-caspase-9-caspase-3 pathway, independent of microsatellite instability status.	(18)
Martinet (2025)	Electrosurgical Cold Plasma Device/APC 3 VIO 3	Argon	"Cold mode" operation generates ROS (especially H_2O_2) and induces dose-dependent cytotoxicity.	(22)

aims to: (1) characterize the voltage- and frequency-dependent generation of H_2O_2 by our tunable argon plasma jet; (2) quantify the resulting cytotoxicity in the chemoresistant HT29 cell line; and (3) establish a predictive model linking the H_2O_2 dose to cell death, thereby demonstrating a novel strategy to bypass a well-defined molecular resistance mechanism in CRC.

Materials and Methods

Plasma Jet Design and Power Supply

A custom-fabricated argon plasma jet was developed to generate a spatially uniform and stable nonthermal plasma plume for RONS delivery. The jet body, constructed from acrylonitrile butadiene styrene (ABS), housed a quartz dielectric tube (3 mm inner diameter, 5 mm outer diameter). A stainless-steel rod (1.8 mm diameter), serving as the high-voltage electrode, was inserted into the quartz tube. A 5 mm-long grounded copper ring electrode was positioned on the tube's exterior, such that its distal edge was 4 mm from the tube exit (Figure 1). This coaxial electrode configuration, with the central electrode aligned within the axial plane of the ring, ensured a symmetrical and consistent plasma discharge. The system operated with 99.999% pure argon, regulated at 2 standard liters per minute (SLM) using a mass flow controller. Plasma excitation was achieved using a custom-built kHz AC power supply (1–20 kV, 18–28 kHz) with independent voltage and frequency tuning. Electrical diagnostics identified a resonant frequency range of 20–24 kHz, corresponding to maximum energy transfer to the plasma column.

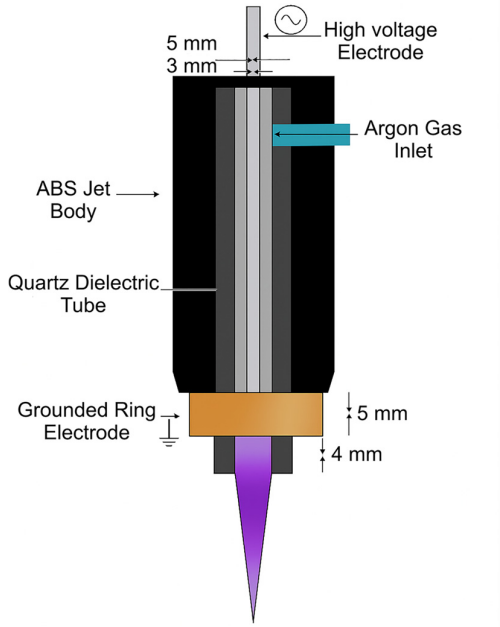

Figure 1. Schematic representation of the Argon Plasma Jet nozzle.

Cell Culture

The human colorectal adenocarcinoma cell line HT29 (IBRC, C10097) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. For experiments, cells in the logarithmic growth phase were detached using 0.25% trypsin-EDTA, and seeded in 35 mm culture dishes at a density of 2×10^5 cells/dish in 2 mL of complete medium. After 24 hours of incubation, cultures reached 70–80% confluency.

Plasma Treatment

Prior to treatment, HT29 cells cultured in DMEM with 10% heat-inactivated FBS were washed twice with Phosphate-buffered saline (PBS). For viability assays, cells were incubated in 2 mL of serum-free DMEM to avoid FBS-mediated scavenging of plasma-generated reactive species. For H_2O_2 quantification, 2 mL of DMEM (without FBS) was treated under identical conditions in cell-free dishes. The plasma jet nozzle was positioned 10.0 mm vertically above the liquid surface (Figure 2). Cells were exposed to argon plasma at seven distinct voltage-frequency combinations during the screening phase, including (9.5 kV, 18 kHz), (10.5 kV, 18 kHz), (9.5 kV, 25.5 kHz), (10.5 kV, 25.5 kHz), (11.1 kV, 25.5 kHz), (9.5 kV, 28 kHz), and (10.5 kV, 28 kHz) , each applied for 3 minutes. For time-dependency analysis, the optimized parameters of (10.5 kV, 28 kHz) were applied for 1 to 4 minutes. Argon (99.999% purity) continuously supplied at 2 SLM. Four hours after plasma exposure,

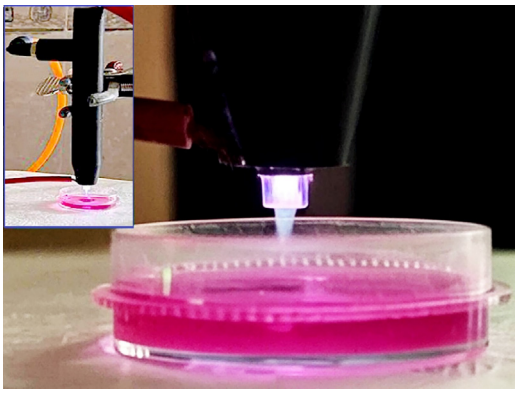

Figure 2. Argon plasma jet treatment in a 35 mm culture dish.

0.22 mL of heat-inactivated FBS was added to restore 10% serum concentration, standardizing post-treatment conditions for viability assays. All experiments were conducted in triplicate biological replicates, with untreated serum-free controls processed in parallel.

Cell Viability Assay (MTT)

Cell viability was assessed 24 hours post-treatment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After plasma exposure, cells were incubated with 0.5 mg/mL MTT in PBS for 4 hours at 37°C under 5% CO_2. The supernatant was gently removed, and the resulting formazan crystals were solubilized in 1 mL dimethyl sulfoxide (DMSO). Absorbance at 570 nm was recorded using a spectrophotometric microplate reader. All measurements were performed in triplicate.

H_2O_2 Concentration Measurement

H_2O_2 concentrations in cell-free DMEM (2 mL) were quantified using a colorimetric hydrogen peroxide assay kit (ZellBio GmbH) following the manufacturer's protocol. All plasma treatment conditions were replicated identically in the quantification assays.

Statistical Analysis

Statistical analyses were performed using Origin 10.

Results

Voltage- and Frequency-Dependent Cytotoxicity

Systematic modulation of voltage (9.5–11.1 kV) and frequency (18–28 kHz) revealed distinct cytotoxic profiles in HT29 cells following 3- minute exposure to the argon plasma jet (Figure 3). At 18 kHz, increasing the voltage from 9.5 kV to 10.5 kV reduced viability from 25.16% ± 3.9% to 19.87% ± 3.2%, demonstrating voltage-dependent enhancement in cytotoxicity. In contrast, at 25.5 kHz, all tested voltages (9.5, 10.5, 11.1 kV) yielded comparatively higher viability rates (viability: 35–48%), suggesting frequency-mediated attenuation of reactive species generation. Notably, Maximum cytotoxicity (9.2% ± 3.6% viability) was observed at 10.5 kV and 28 kHz, while under identical frequency, reducing the voltage to 9.5 Kv resulted in lower cytotoxicity (54.3% ± 1.2% viability). These findings underscore the critical interplay between voltage and frequency in modulating CAP-induced cell death.

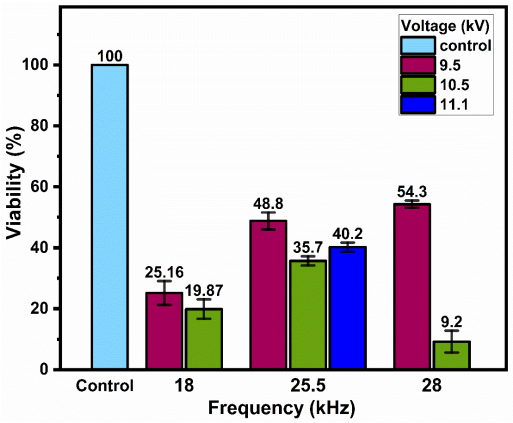

Figure 3. Voltage- and frequency-dependent cytotoxicity in HT29 cells after 3-minute plasma treatment. MTT assay results show cell viability (%) at different voltage-frequency combinations (9.5–11.1 kV, 18–28 kHz). Maximum cytotoxicity (9.2% viability) occurred at 10.5 kV, 28 kHz. Data: mean ± SD (n=3).

Time-Dependent Cytotoxicity at Optimal Parameters

Time-course experiments conducted at the optimized conditions (10.5 kV, 28 kHz) demonstrated rapid and saturable cytotoxic kinetics in HT29 cells (Figure 4a). Cell viability declined exponentially from 91.4% ± 3.9% after 1 minute of plasma exposure to 9.2% ± 3.6% after

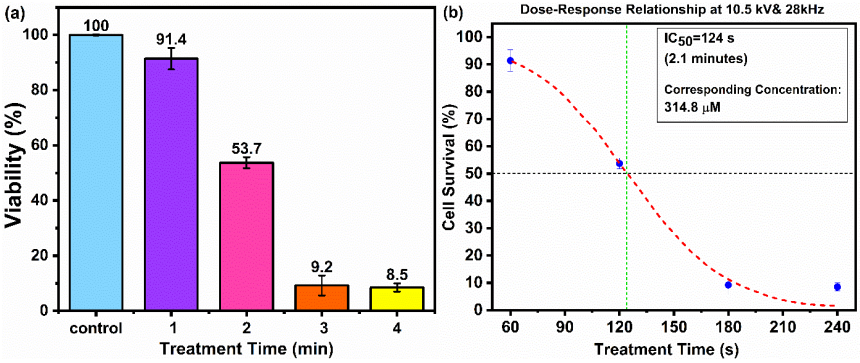

Figure 4. Time-dependent cytotoxicity at 10.5 kV, 28 kHz. **a**. Viability decreased progressively with exposure time (1–4 minutes). Data: mean ± SD (n=3). **b**. Correlation between H_2O_2 concentration and IC50 at 10.5 kV, 28 kHz. The IC50 treatment time (124 seconds) corresponds to 314.8 μM H_2O_2.

3 minutes, with an IC_{50} of 124 s (Figure 4b). Extending the exposure to 4 minutes resulted in 8.5% ± 1.5% viability, showing no statistically significant increase in cytotoxicity beyond the 3-minute mark. This plateau suggests that H_2O_2 accumulation may reach saturation, limiting additional cytotoxic effects despite prolonged treatment duration.

Tunable H_2O_2 Generation via Voltage-Frequency Modulation

Extracellular H_2O_2 concentrations measured in cell-free DMEM closely mirrored the observed cytotoxicity patterns. At 10.5 kV, H_2O_2 production reached its peak value of 371.0 ± 12.7 µM (at 28 kHz), surpassing the levels obtained at 25.5 kHz (319.15 ± 12.09 µM) and 18 kHz (347.75 ± 14.50 µM) (Figure 5). Frequency modulation produced distinct H_2O_2 generation profiles that were strongly dependent on the applied voltage (Figure 6). Specifically, at 10.5 kV, H_2O_2 concentrations followed a parabolic response, with a clear maximum at 28 kHz (Figure 7a). In contrast, at 9.5 kV, H_2O_2 output exhibited an exponential decay with increasing frequency, declining from 335.94 ± 16.59 µM (at 18 kHz) to 291.50 ± 9.19 µM (at 28 kHz) (Figure 7b). Notably, operation at 11.1 kV, 25.5 kHz yielded suboptimal H_2O_2 levels (310.55 ± 13.36 µM), possibly due to reactive species quenching under excessively energetic plasma conditions.

Temporal Kinetics of H_2O_2 Accumulation

Under optimized plasma conditions (10.5 kV, 28 kHz), H_2O_2 accumulation in cell-free DMEM exhibited time-dependent saturation behavior (Figure 8). The concentration increased progressively, reaching 371.0 ± 12.7 µM at 3 minutes, which corresponded closely with the observed IC_{50} value of 314 µM for HT29 viability. Extending plasma exposure to 5 minutes resulted in only a modest further increase in H_2O_2 levels (385.0 ± 16.5 µM), indicating a plateau phase.

H_2O_2 Drives Argon Plasma Jet-Induced Cytotoxicity

A strong, statistically significant linear correlation ($R^2 = 0.995$, $p < 0.001$) was observed between extracellular H_2O_2 concentrations (ranging from 291 to 371 µM) and HT29 cell viability across all tested voltage–frequency conditions (Figure 9). Regression analysis confirmed that H_2O_2 levels alone could reliably predict treatment-induced cytotoxicity, highlighting the potential for dose-controlled oxidative stress induction via precision tuning of the khz-driven argon plasma jet.

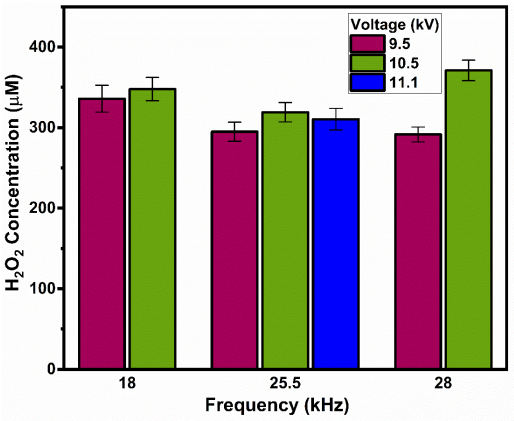

Figure 5. H_2O_2 generation across voltage-frequency combinations. H_2O_2 concentrations (µM) in cell-free DMEM after 3-minute exposure. Highest H_2O_2 (371.0 ± 12.7 µM) at 10.5 kV, 28 kHz. Data: mean ± SD (n=3).

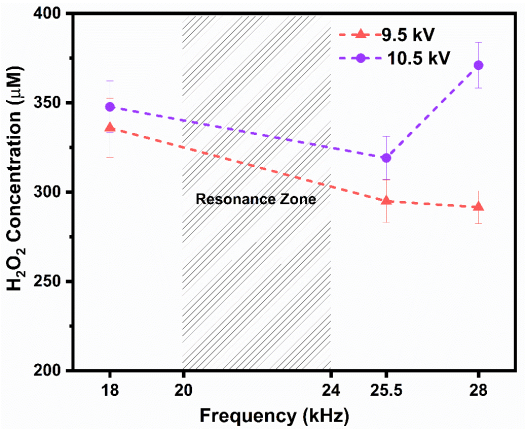

Figure 6. H_2O_2 concentration for 9.5 kV and 10.5 kV across frequency. At 10.5 kV, H_2O_2 generally exceeded 9.5 kV. Data: mean ± SD (n=3).

Discussion

This study demonstrates that a voltage- and frequency-tunable argon plasma jet can overcome chemoresistance in HT29 CRC cells through the precise, dose-controlled delivery of H_2O_2. Our principal finding is the exceptionally strong linear correlation ($R^2 = 0.995$, $p < 0.001$) between the extracellular H_2O_2 concentration and the reduction in cell viability. By optimizing the electrical parameters to 10.5 kV and 28 kHz, we generated a cytotoxic environment that reduced HT29 viability to just 9.2%, showcasing a level of efficacy not easily achieved with conventional, non-tunable plasma systems against this resistant cell line.

Overcoming the Antioxidant Defense

The significance of our findings is best understood in

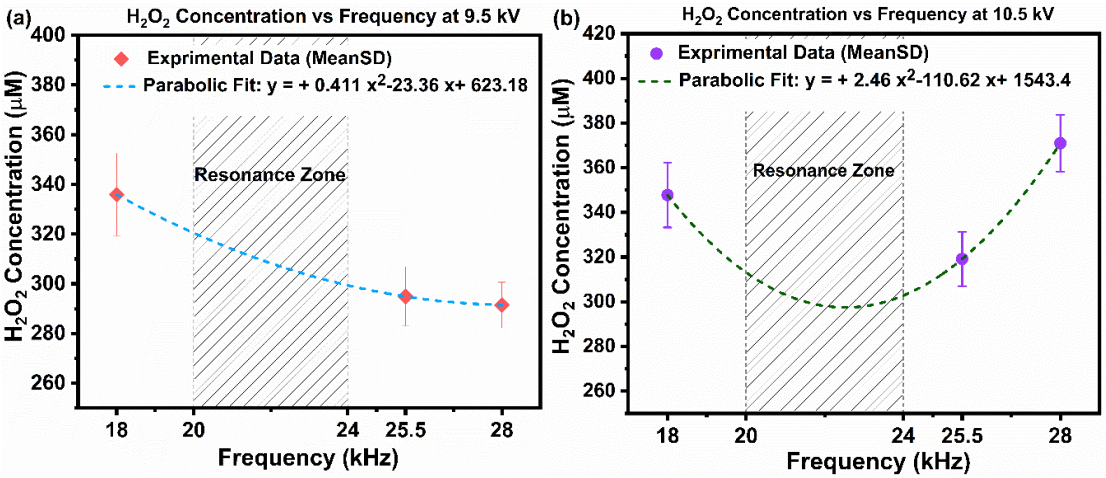

Figure 7. Frequency-dependent H_2O_2 trends. **a** At 9.5 kV, parabolic .**b** At 10.5 kV, parabolic. Data: mean ± SD (n=3).

the context of HT29's known resistance mechanisms. These cells exhibit a high basal expression of the Nrf2/Srx antioxidant axis, a protective system that effectively neutralizes ROS-induced damage and confers resistance to both chemotherapy and certain forms of CAP (17, 23-29). The work by Ishaq et al. (2014) was pivotal in showing that a helium plasma jet failed to induce significant apoptosis in HT29 cells unless the Nrf2/Srx pathway was silenced via siRNA (17). In stark contrast, our tunable argon plasma jet induces potent cytotoxicity without any genetic or chemical sensitization. We propose that by precisely tuning the voltage and frequency, we can generate a sufficiently high and sustained flux of H_2O_2 (up to 371 µM) that effectively saturates and overwhelms the buffering capacity of the Nrf2/Srx system. This ability to overcome a specific, well-documented resistance pathway represents a key novelty and a significant step forward for plasma cancer therapy.

The Interplay of Voltage and Frequency in Controlling Plasma Chemistry

The experimental data revealed a clear interplay between applied voltage and driving frequency in modulating H_2O_2 production within the argon plasma jet. Voltage dependence was evident, as higher voltages (e.g., 10.5 kV compared to 9.5 kV) promoted electron-impact reactions, leading to an increased •OH radical density ($Ar + e^- \rightarrow Ar^* + \bullet OH$) and subsequent H_2O_2 formation ($\bullet OH + \bullet OH \rightarrow H_2O_2$) (30). Our results indicate the existence

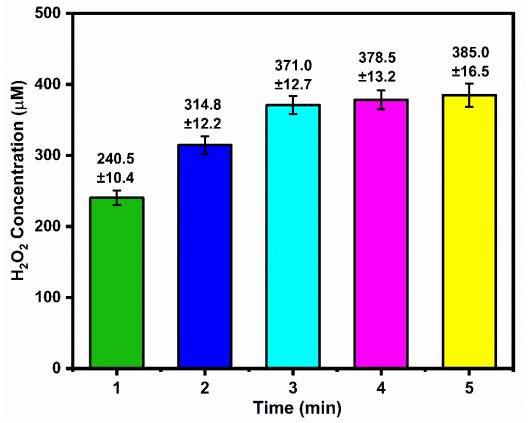

Figure 8. Time-dependent H_2O_2 accumulation at 10.5 kV, 28 kHz. Saturating increase in H_2O_2 (1–5 minutes). Plateau was observed after 3 minutes. Data: mean ± SD (n=3).

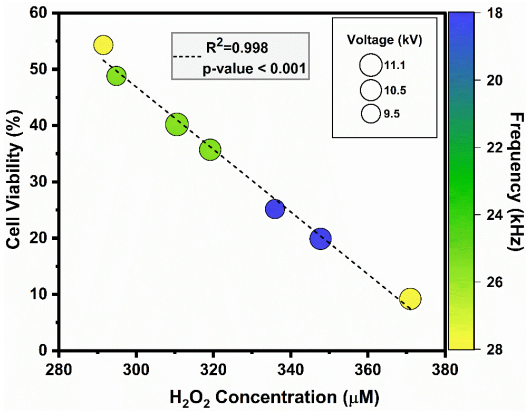

Figure 9. Linear correlation between H_2O_2 concentration and HT29 cell viability. Strong inverse relationship (R^2 = 0.995, $p < 0.001$).

of an optimal 'sweet spot' that maximizes the net production of H_2O_2 by balancing its formation and decomposition pathways. While higher voltage increases the energy for •OH formation, excessively high energy input, such as at 11.1 kV, can become counterproductive by enhancing H_2O_2 decomposition pathways (e.g., $H_2O_2 + e^- \longrightarrow 2•OH$), leading to a net decrease in its concentration (31). The optimal point at 10.5 kV and 28 kHz likely represents the ideal balance where electron energy is sufficient for robust •OH formation without excessively promoting these decomposition reactions. Frequency tuning further influenced this balance: operating at 28 kHz, beyond the system's resonant range (20–24 kHz), maximized H_2O_2 generation, likely by optimizing the pulse repetition rate to favor formation over decomposition processes (32).

The Importance of a Tunable Power Supply and Advantages over RF/DBD Sources

Plasma jets are commonly powered by a range of electrical sources, such as RF generators, pulsed DC supplies, and kHz-driven AC systems (33). The choice of a kHz AC power supply was deliberate and central to our hypothesis. The key advantage of this system over most RF power supplies (which operate at fixed frequencies) is the independent control over both voltage and frequency. Our data clearly show that the optimal H_2O_2 production is highly dependent on a specific, non-linear combination of these two parameters, a level of control fixed-parameter systems cannot offer. Furthermore, our jet configuration offers distinct advantages over DBD sources for internal tumors like CRC. DBDs are best suited for surface treatments , whereas our plasma jet produces a focused plume that can be guided down an endoscope to precisely target a tumor without invasive electrode contact (34). Critically, the therapeutic effect of our argon jet is mediated primarily by long-lived aqueous species (H_2O_2) generated at the plasma-liquid interface, which is ideal for the fluid-covered physiological environment of the colon (35). This contrasts with many DBDs whose efficacy relies more on short-lived species, intense electric fields, and UV radiation, which are less suitable when a significant liquid layer is present (35). Recent research continues to explore the unique chemical and biological effects that can be achieved with kHz-driven plasma sources, underscoring the importance of this technological approach for advancing plasma medicine (36).

The Unique Advantage and selectivity of CAP

A fundamental advantage of CAP in oncology is its inherent selectivity for cancer cells over healthy cells (37-40). This selectivity is primarily rooted in the distinct redox biology of malignant cells. Cancer cells exist in a state of chronic oxidative stress with elevated basal levels of intracellular ROS, placing them much closer to an apoptotic threshold than their healthy counterparts (41). Therefore, a precisely tuned dose of H_2O_2, like the one delivered by our system, is sufficient to overwhelm cancer cells, pushing them into apoptosis. This principle ensures that the therapeutic dose remains within the buffering capacity of adjacent healthy tissue, maintaining a high therapeutic window (42).

The strong correlation between H_2O_2 concentration and cell viability raises a critical question: why is the complexity of CAP necessary if H_2O_2 is the principal cytotoxic agent? The efficacy of CAP is not attributable to H_2O_2 alone but to the synergistic interplay of its diverse chemical and physical components (33). Although our data identify H_2O_2 as the dominant long-lived species and the most reliable predictor of cytotoxicity in this argon jet system, it does not act in isolation. It is well-established that H_2O_2 applied alone cannot fully replicate the potent and selective cytotoxicity of plasma-activated medium (PAM) (43), and even synergistic mixtures of $H_2O_2/NO_2^-/NO_3^-$ fall short of matching its biological efficacy (44, 45). Therefore, the primary advantage of our plasma jet is its ability to deliver a complex, synergistic "therapeutic cocktail," while the tunability provides precise control over the dose of its key long-lived effector, H_2O_2.

Clinical Applicability and Implications for Precision Plasma Oncology

Furthermore, the exceptionally strong linear correlation ($R^2 = 0.995$) established in this study has profound implications for the development of 'precision plasma oncology'. This relationship validates extracellular H_2O_2 concentration as a simple and reliable predictive biomarker for the biological effect of our argon plasma jet. It provides a quantitative link between tunable physical parameters (voltage, frequency) and a predictable cytotoxic outcome. This opens the door to personalized therapeutic strategies where: (1) a patient's tumor can be profiled for its specific antioxidant capacity; (2) a required cytotoxic H_2O_2 dose can be prescribed; and (3) the plasma jet can be precisely tuned to deliver that exact dose, maximizing efficacy while minimizing off-target effects. This paradigm shifts plasma therapy from a qualitative application to a quantitative, dose-controlled, and personalized treatment modality.

Limitations and Future Directions

We acknowledge several limitations in this study. Our investigation was performed on a 2D in vitro model,

which does not capture the complexity of the tumor microenvironment. While we identified H_2O_2 as the primary driver of cytotoxicity, we did not quantify other RONS or investigate detailed molecular pathways. Future work should validate these findings in more clinically relevant models, such as 3D tumor spheroids and in vivo orthotopic CRC models. Furthermore, exploring synergistic combinations of this dose-controlled plasma treatment with standard chemotherapeutics could lead to more effective, lower-dose treatment regimens (46). Finally, scaling and miniaturization of the plasma jet for endoscopic application will be crucial for its eventual clinical translation.

Conclusion

This study demonstrates that a custom-engineered, voltage- and frequency-tunable argon plasma jet is a highly effective platform for overcoming intrinsic chemoresistance in HT29 colorectal cancer cells. The central finding is the establishment of a precise, dose-controlled delivery of H_2O_2 by systematically tuning the plasma's electrical parameters. Optimal settings (10.5 kV at 28 kHz) maximized cytotoxicity, reducing cell viability to just 9.2%. This effect was underpinned by a strong linear correlation ($R^2 = 0.995$) between H_2O_2 concentration and cell death, establishing H_2O_2 as a robust predictive biomarker for therapeutic outcome. Crucially, this approach circumvents the redox resistance without sensitization, offering a blueprint for personalized, parameter-driven plasma oncology.

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