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Innovative Therapeutic Approach Targeting Colon Cancer Stem Cells: Transitional Cold Atmospheric Plasma

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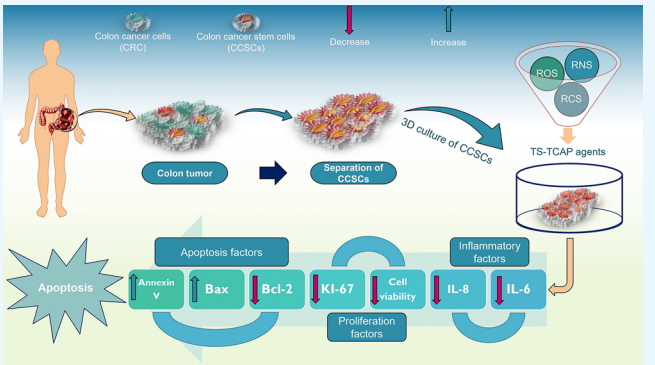
ABSTRACT: Transitional cold atmospheric plasma (TCAP) represents a novel technique for generating plasma remotely from a primary source. It consists of a partially nonthermal ionized gas mixture containing charged and neutral particles, photons, and free radicals. In recent years, TCAP has attracted considerable attention in biomedical applications. In order to evaluate colon cancer stem cells' (CCSCs) proliferation, apoptotic induction, inflammatory response, and survival, TCAP was utilized both directly and indirectly in this study. Using argon and helium gases, TCAP was continuously delivered in two stages during the experiment. For direct state, TCAP was irradiated onto CCSCs for 3 and 5 min. In the indirect technique, Matrigel was treated with TCAP for 5 min before the introduction of cells. In vitro assays demonstrated that TCAP exposure significantly reduced the viability of CCSCs; helium gas and direct application had greater impacts than argon. Numerous investigations confirmed the induction of apoptosis, showing that the treated groups had more apoptotic cells and altered cellular structures than controls (****$p < 0.0001$). A substantial increase in the Bax/Bcl-2 ratio was found by analyzing the expression of the Bax and Bcl-2 genes, indicating increased susceptibility to apoptosis (*$p = 0.0177$ and ***$p = 0.0004$). The higher efficacy of the direct helium mode was further highlighted by inflammatory marker analysis, which showed a significant reduction in interleukin-6 and interleukin-8 expression in cells directly treated with TCAP-helium compared to TCAP-argon (**$p = 0.0015$ and ***$p = 0.0007$). Lastly, the proliferation test, which relies on K_i-67 expression, demonstrated a noteworthy decline in all TCAP-treated groups, with the direct helium group exhibiting the most robust impact (**$p = 0.0014$). Overall, the findings highlight the potential of TCAP, particularly with helium, as a promising approach for selectively targeting CCSCs and providing insights into its therapeutic mechanisms for cancer treatment. TCAP, therefore, emerges as a unique therapeutic strategy with potential applications in cancer stem cell-targeted therapies.



1. INTRODUCTION

1.1. Background Information. Colorectal cancer (CRC), the third most common cause of cancer-related mortality worldwide, is a highly heterogeneous disease characterized by substantial molecular and genetic diversity.[1,2] The malignancy arises due to uncontrolled cellular proliferation and enhanced invasiveness, driven by phenotypic alterations and disruptions in cellular survival mechanisms, culminating in the formation of colon tumors.[3,4] Despite advancements in conventional treatments, including surgical intervention, chemotherapy, and radiotherapy, the prognosis for early stage colon cancer remains poor.[5] One significant barrier to successful treatment outcomes is the presence of cancer stem cells (CSCs), specifically colon cancer stem cells (CCSCs).[6] These are a small subset of cells with stem cell-like properties that play a pivotal role in tumor initiation and progression.[7] Unlike normal stem cells, CCSCs are thought to arise from damaged colonic epithelial stem cells.[8] They possess the ability to generate differentiated progeny while sustaining growth, which contributes to cancer initiation, progression, recurrence, and resistance to standard therapies.[9,10] Key characteristics of CCSCs include self-renewal, plasticity, tumorigenicity, high metastatic potential, and the expression of specific surface markers.[11,12] Biomarkers such as CD133,[13] CD44,[14] CD166,[15] and aldehyde dehydrogenase 1 (ALDH1)[16] are used to distinguish CCSCs from other intestinal cells. CCSCs exhibit robust self-renewal capacity, supported by the inhibition of apoptosis via the Notch signaling pathway, which suppresses the cell cycle inhibitor P27.[17,18] Additionally, the inherent drug resistance of CCSCs is a key factor contributing to tumor persistence and recurrence following treatment, rendering traditional modalities like chemotherapy and radiotherapy less

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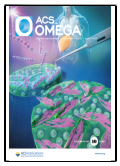

effective.[19,20] Consequently, targeting CCSCs has become a critical focus in cancer therapy research.[21]

Emerging therapeutic approaches aim to address the limitations of existing treatments.[22] Among these, cold atmospheric plasma (CAP) has garnered significant attention as a novel, noninvasive therapeutic strategy.[23] CAP is a nonthermal, partially ionized gas mixture comprising neutral and charged particles, photons, and reactive species, generated under atmospheric pressure and room-temperature conditions.[24,25] CAP can be applied directly to biological targets or indirectly through plasma-activated media (PAM), where a liquid exposed to plasma is subsequently introduced to the target.[26−28] Matrigel, a gelatinous protein mixture derived from mouse sarcoma cells, can serve as a PAM due to its extracellular matrix-like properties, supporting three-dimensional (3D) cell cultures, enhancing cell function, and facilitating disease modeling, drug testing, and toxicological studies.[29−33] CAP can be generated through various methods, including atmospheric pressure plasma jets (APPJ) and dielectric barrier discharge (DBD) systems.[34] More recently, transported cold atmospheric plasma (TCAP) has emerged as a promising tool in medical applications.[35] TCAP involves the delivery of plasma from a primary source via capillary tubes, enabling the safe and effective transport of plasma-generated reactive components to biological targets.[36,37]

The biomedical applications of CAP are diverse, encompassing sterilization,[38] microbial decontamination,[39] wound healing,[40] blood coagulation,[41] dentistry,[42] and cancer therapies.[43] CAP has demonstrated anticancer efficacy in various tumor models, including melanoma,[44] breast cancer,[45] liver cancer,[46] bone sarcoma,[47] cervical cancer,[48] lung adenocarcinoma,[49] thyroid carcinoma,[50] oral carcinoma,[51] and colorectal cancer.[52] Importantly, CAP exhibits selectivity in its anticancer effects, effectively targeting malignant cells while sparing normal tissues.[53] While the precise mechanisms underlying CAP's antitumor activity remain under investigation,[54] its ability to generate reactive oxygen and nitrogen species (RONS) plays a crucial role.[55] These reactive species induce oxidative stress in cancer cells,[56] triggering intracellular signaling cascades that result in apoptosis,[57] cell destruction,[58] and reduced drug resistance.[59] By enhancing oxidative stress and activating specific cellular and molecular pathways, CAP also stimulates the immune response, ultimately promoting cancer cell death.[60]

1.2. Research Problem. *1.2.1. Current Challenges in Colon Cancer Therapy.* Conventional therapies for colon cancer often fail to achieve complete remission due to the persistence of CSCs.[61] These cells' improved DNA repair skills, increased expression of drug efflux transporters, and quiescent state all contribute to their exceptional resistance to radiation and chemotherapy.[62] Furthermore, CSCs are essential for maintaining tumor heterogeneity and promoting metastasis, making them a key target for long-lasting therapeutic effects.[63] The essential need for new and creative treatment approaches is highlighted by the incapacity of current treatments to completely eradicate CSCs.[64]

1.2.2. Potential of Cold Atmospheric Plasma. TACP involves applying cold plasma—a state of matter composed of charged particles and reactive species—to tissues or tumors.[65] Preclinical research highlights its anticancer properties, including the induction of apoptosis and necrosis, inhibition of inflammatory cytokines, and suppression of cancer cell growth through RONS production.[66]

TACP offers several unique advantages in cancer treatment, particularly in targeting cancer cells and CSCs:

- Selective targeting of CSCs: TACP can precisely eliminate CSCs while sparing normal cells, reducing collateral tissue damage often seen in conventional therapies.[67]
- Signaling pathway modulation: TACP disrupts pathways critical for CSC survival and proliferation, inhibiting their regenerative capabilities within tumors.[68]
- Enhanced sensitivity to other therapies: TACP increases CSC susceptibility to chemotherapy and radiation, improving the effectiveness of combined treatments.[69,70]
- Non-thermal and non-invasive nature: unlike traditional procedures, TACP is noninvasive and does not rely on heat, allowing for external or minimally invasive applications that reduce patient recovery time and discomfort.[71]
- Versatility and safety: TACP can be tailored to target specific cancer types and tumor regions, enhancing precision while ensuring safety, such as minimizing electric shock risks.[35]
- Synergistic potential: TACP can complement emerging therapies, like immunotherapy and targeted treatments, to address multiple aspects of cancer progression simultaneously.[72]

1.3. Mechanistic Hypothesis. *1.3.1. Hypothesis.* We hypothesize that TCAP selectively targets CCSCs due to its remarkable efficiency and safety profile, effectively disrupting critical signaling pathways necessary for their survival and self-renewal. This dual-action mechanism enhances therapeutic efficacy while minimizing the risk of tumor recurrence. Specifically, we hypothesize that TCAP-induced RONS lead to oxidative stress, triggering apoptotic cell death in CCSCs. Furthermore, TCAP-mediated suppression of inflammatory and proliferative signaling pathways further diminishes the functional capacity of CCSCs.

1.3.2. Rationale.

- Enhanced susceptibility of CCSCs to oxidative stress: CCSCs depend on a finely tuned redox equilibrium to sustain their viability and stem-like properties. RONSs generated by CAP have been demonstrated to induce oxidative stress in cancer cells. This disruption can overwhelm the antioxidant defenses of CCSCs, potentially leading to their targeted elimination.
- Induction of apoptosis: the dysregulation of apoptosis in CCSCs is a critical factor in tumor initiation, progression, and therapy resistance. Emerging evidence suggests that CAP can modulate key apoptotic regulators, including Bax and Bcl-2 proteins, within CCSCs, thereby promoting programmed cell death and reducing tumor survival.
- Suppression of inflammatory mediators: pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and interleukin-8 (IL-8), play essential roles in the pathogenesis of colon cancer by supporting the stemness and tumorigenic capacity of CCSCs. CAP treatment has been shown to downregulate the expression of these cytokines, thereby mitigating the inflammation-driven progression of colon cancer.
- Enhancing CAP safety and efficiency: the utilization of capillary tubes for plasma delivery enhances both the

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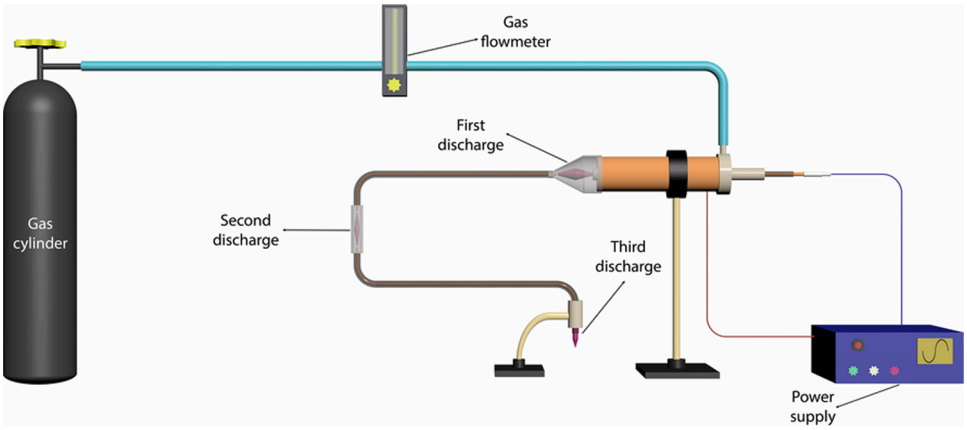

Figure 1. TCAP configuration schematic.

safety and efficacy of CAP therapy by reducing voltage and optimizing the production of RONSs.

1.3.3. Predictions.

- TCAP therapy decreases CCSC viability in vitro: TCAP administration significantly reduces the survival and viability of CCSCs.
- TCAP enhances apoptosis-related gene expression in CCSCs: TCAP enhances programmed cell death in CCSCs by favorably influencing the expression of important genes linked to apoptotic pathways.
- TCAP reduces the expression and secretion of inflammatory cytokines in CCSCs: TCAP exposure decreases the inflammatory milieu that promotes tumor growth by preventing the synthesis and release of pro-inflammatory cytokines.
- TCAP inhibits proliferative factors in CCSCs: TCAP therapy successfully lowers the expression of factors linked to CCSC proliferation, which limits the cells' capacity to promote tumor development and migration.

1.3.4. Objectives and Aims. 1.3.4.1. Primary Objective. Investigation into the influence of TCAP exposure on the biological behaviors of CCSCs.

1.3.4.2. Specific Aims. Aim 1: assess TCAP's treatment effectiveness in CCSCs

- Evaluate the effect of TCAP therapy on the CCSC's viability.
- Examination of TCAP therapy in the CCSCs apoptotic induction.
- The impact of TCAP therapy on CCSC proliferation.
- Investigation of TCAP therapy on the inflammatory factors of CCSCs

Aim 2: examine the potential and safety of TCAP

Evaluation of TCAP treatment's safety in CCSCs.
Evaluation of possible clinical use of TCAP as a unique therapeutic approach that targets CCSCs, including treatment regimen and delivery system optimization.

2. MATERIALS AND METHODS

2.1. Materials. *2.1.1. CCSCs.* The HT29 cell line cultured CCSCs (from the NCBI cell bank, Pasteur Institute of Iran), Matrigel matrix (Corning, Arizona, USA), 96-well microplates (Nunc, Roskilde, Denmark), 48-well plates (Shanghai Biotechnology, China), streptomycin, DMEM/F12 medium, B27 supplement (Gibco, USA), recombinant human epidermal growth factor (Sigma, USA), basic fibroblast growth factor (Upstate Products, USA), leukemia inhibitory factor, insulin (Sigma, USA), vincristine (Shanghai Hualin Pharmaceutical Co., China), an ELISA plate reader (model XYZ, Roche Applied Sciences, Indianapolis, USA), and the RNA PCR Kit 3.0 (TaKaRa, Japan) are equipment and materials utilized.

2.1.2. TCAP. The materials and equipment utilized in the production of TCAP included a power source (Basafan, Tehran, Iran), argon and helium gases (Soheil Gas, Karaj, Iran), a quartz tube (Avijeh, Tehran, Iran), PTFE polymer (Pars Polymer, Tehran, Iran), copper electrodes (Bahonar, Kerman, Iran), and plastic pipes (Sabra, Tehran, Iran).

2.2. Methods. Examining TCAP's direct and indirect impacts on the biological components of CCSCs grown in a 3D culture medium was the aim of this investigation. Using argon and helium as working gases, an AC voltage was used to generate the TCAP that was utilized. Human CCSCs were grown in a three-dimensional culture for experiments. TCAP was applied directly to CCSCs for a duration of three and 5 min in the direct state. Prior to the addition of cells in the indirect approach, Matrigel was treated with TCAP for 5 min. Following treatment with TCAP, cells were subjected to extensive investigations, which included histological, electron microscopy, and biological evaluations. These investigations comprised flow cytometry, biochemical analysis, RT-PCR, ELISA, and the assessment of apoptosis-related protein and enzyme expression.

2.2.1. Cell Culture in Matrigel. CCSCs cultivated in RPMI-1640 medium fortified with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). The experiment was conducted in a 37 °C moistened incubator with 95% air and 5% CO_2. A prechilled 48-well plate was used for plate preparation, and 200 μL of Matrigel matrix was applied to each well. The Matrigel matrix was allowed to gel on the plate by incubating it at 37 °C for 30 min, followed by spreading with a pipet tip. Then, for 14−18 h, CCSCs were inserted with 2×10^4 cells/well in 48-well plates utilizing a cultivation ambience comprising 500 μL Dulbecco's Modified Eagle Medium, 10% FBS, 100 U/mL penicillin G, and 100 mg/mL streptomycin. Subsequently, 250 μL of the cultivated ambience was substituted with a corresponding volume of serum-free cancer stem cell ambience. DMEM/F12, leukemia inhibitory factor (10 ng/mL), basic fibroblast growth factor (20 ng/mL), recombinant human epidermal growth factor (20 ng/mL), B27, insulin (4 U/L), and vincristine (5 ng/mL) are

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the components of this ambience. The process was carried out once again.

2.2.2. TCAP. The plasma generation in this study follows a three-stage process, illustrated in Figure 1. In the initial stage, by applying high voltage (HV), plasma is produced within a cylindrical coaxial dielectric barrier discharge (DBD) reactor, comprising internal and external electrodes. The external electrode is a 24 mm diameter, 2 mm thick copper tube, whereas a copper wire with a diameter of 2 mm as the interior electrode is situated within a 4 mm diameter, 1 mm thick quartz glass pipe. These electrodes are separated by a polytetrafluoroethylene (PTFE) polymer insulator, and the quartz tube with the internal electrode is placed inside it. The principal plasma was generated in the DBD reactor by applying a high voltage to the interior electrode. In the second stage, the end of the external electrode is connected to the first plastic transfer tube (5 m in length and 6 mm in external diameter) via a funnel-shaped Pyrex glass. Inside this transfer pipe, a slender copper wire (0.1 mm in diameter) acts as the main transmission agent, starting near the PTFE insulation and extending to the tube's end. By inducing an electric field at its terminus, this copper wire transfers the primary plasma from the DBD reactor to the end of the first tube, giving rise to an APPJ at the first transfer tube's exit. The third stage involves connecting a second plastic transfer tube (matching the length and thickness of the first transfer tube) to the first transfer tube through a Pyrex glass tube. Similar to the first plastic tube, a thin copper wire inside the second transfer tube, slightly inserted into the glass tube, serves as the secondary factor for transferring the APPJ ignited from the end of the first transmission pipe to the secondary transfer pipe's end. Ultimately, another APPJ is formed at the secondary transfer pipe's terminus, constituting the TCAP.

The power source was an AC half-bridge with varying frequencies. This setup provided an output voltage of 18 kVp-p with a maximum output current of 500 mA. The spectrometer employed for optical emissions diagnostics was the Ocean Optics USB4000, covering a range of 375−1100 nm, and the exposure time was set to 1000 ms. Finally, to collect electrical data from TCAP, a Tektronix TDS2024D oscilloscope was used with two AOP-10 HV probes (500 MΩ, 1000×, 3 pF, 10 MHz), along with a conventional 10× probe.

2.2.3. In Vitro Assay of Cell Viability. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) approach was used to investigate the direct as well as indirect impact of TCAP on CCSC's survivability. MTT tests were conducted to gauge the cell vitality in various groups of cultured CCSCs, with each experiment performed in triplicate. 5×10^3 cells were planted onto a sterile scaffold in each well of 96-well microplates. In order to promote the adhesion of CCSCs to the well surfaces, 5 mg/mL from the stock concentration of MTT was given to each well after 24, 48, and 72 h of culture. The wells were then incubated for 24 h at 37 °C in a humid environment with 5% CO_2. The regular culture medium and CCSC-filled control wells were kept in place. Then, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals that had formed after the cultivation environment, and any leftover MTT solution was eliminated from the wells. After that, the absorbance was determined using an ELISA plate reader at a wavelength of 570 nm. Cell viability was determined using the following formula, which revealed a difference in the survival rate between the control group and the experimental groups:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

2.2.4. RT-PCR. RT-PCR was utilized for the evaluation of the gene expression of the ATP-binding cassette (ABC) transporters, which are in charge of vincristine efflux. Following the manufacturer's instructions, total RNA was extracted from HT29 cells, including adherent nonsphere-forming cells and tumor spheres, using Tripure reagent. For RT-PCR, TaKaRa RNA PCR kit 3.0 was utilized. Each gene was amplified 29 times, with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene acting as an internal control. Following this, each RT-PCR aliquot was electrophoresed in a 1.8% agarose gel that included 0.5 mg per milliliter of ethidium bromide.

2.2.5. Flow Cytometry. Using a flow cytometry method and dual staining with propidium iodide (PI) and Annexin V-FITC, the conditions for triggering apoptosis were evaluated. At first, 2.5×10^5 cells per well of 6-well plates were seeded with cells. Following trypsinization, cells from every experimental group were stained for 15 min at 4 °C in the dark using PI and Annexin V-FITC. The proportion of apoptotic cells in the labeled cells was then determined using Flomax software and a Partec flow cytometer.

2.2.6. Measurement of IL-6 and IL-8. The Quantizing ELISA kit was employed to quantify the values of interleukins 6 and 8. Every well in every testing sample had its cell-free culture supernatant gathered, incubated, and its optical density (OD) recorded at 450 nm. The assay has the ability to identify cytokines in quantities as low as 5 ± 7 pg/mL.

2.2.7. Transmission Electron Microscopy (TEM). Secondary spheres that were derived from single cancer stem cells identified in the initial spheres were first fixed in a 1% osmium tetroxide solution and then preserved in a 2.5% glutaraldehyde solution. The samples were then dehydrated using gradients of ethanol and acetone, and they were placed in Epon812 resin. The specimens were thin-sliced and stained with lead citrate and uranyl acetate. The specimens were examined under a LEO 906 transmission electron microscope.

2.2.8. KI-67. The expression of KI-67 was quantified through RT-PCR.

2.2.9. Statistical Analysis. A meaningful threshold of $p < 0.05$ was utilized when comparing treated cells to untreated cells. Every statistic was graphed via GraphPad Prism 9.0 and conveyed as the mean ± standard deviation (SD). Depending on the kind of investigation, a one-way ANOVA with multiple comparisons may have been one of the statistical analyses chosen. Additionally, Tukey's test was employed to compare the data.

3. RESULTS AND DISCUSSION

The use of CAPs in medical applications, especially in the treatment of cancer, has generated considerable interest in recent years.[73] Among the different forms of plasma, transfer plasmas have garnered attention for their distinct advantages, such as their ability to penetrate distant and inaccessible tissues, coupled with the safety offered by distancing the high-voltage source from the treated object.[74,75] This study introduces the TCAP (two-step cold atmospheric plasma) process method for the first time, representing a significant advancement. It employs argon and helium gases to generate plasma via a DBD reactor, followed by a multistage process to transfer the plasma and produce an APPJ. This innovative

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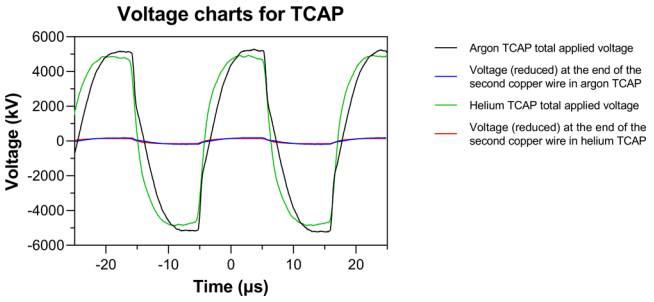

Figure 2. Waveform of the overall voltage applied to the TCAP system and the measured voltage at the end of the second copper wire for argon and helium gases.

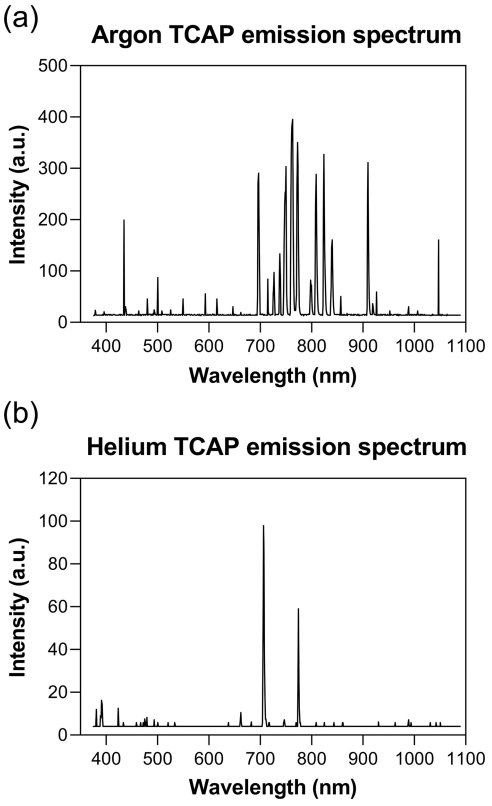

Figure 3. Reactive species produced in TCAP: (a) argon and (b) helium.

approach holds the potential to selectively target CCSCs, inducing apoptosis and cellular damage, while minimizing energy consumption and safety risks.

The TCAP process begins with the introduction of argon and helium gases at flow rates of 4 L/min, with high voltage applied through narrow copper wires in the transfer tubes (11 kV for argon and 10 kV for helium) and corresponding frequencies (40 kHz for argon and 36 kHz for helium). Figure 2 shows graphs of the voltage waveform across the entire TCAP system and the copper wire end. One of the key findings of this study is the significant reduction in voltage observed during the plasma transfer process. The plasma generated in the primary and secondary transfer tubes exhibits a substantial voltage drop compared to the initial high-voltage input, which is likely due to ionization losses. This reduction in voltage not only contributes to a safer environment but also minimizes energy consumption, with power analysis showing that the TCAP system consumes less than 1 W of power for

Table 1. TCAP-Produced Reactive Species

species produced in argon TCAP		species produced in helium TCAP	
species	the wavelengths detected (nm)	species	the wavelengths detected (nm)
Ar I	696.7, 714.7, 727, 738.3, 750.3, 763.3, 772.5, 840, 918.8, 1047.1	He I	706.5, 824.9, 930.4, 962.6, 1031.3
Ar II	434.8 nm	Cu I	479.4
NO	379.1	Cu II	475.3, 533.6, 682.5, 774.4, 808.8
Cu II	808.8, 824, 909.7	Cu III	380.9
O I	615.7, 798, 926.3, 952.4	O I	1042.1
O III	396.2	O II	433.7, 459.1, 467.3, 520.6, 717.6, 861, 993.4
N I	493.5, 646.9, 662.3, 856.7	N I	493.5, 662.3, 746.8, 1050.7
N II	480.4, 500.5, 549.6, 593.1, 988.8, 1006.4	N II	423.6, 500.5, 843.7, 988.8
N III	437.9	N IV	638.1
N^+	508.6	N_2^+	391.1
N_2^+	463.4		
O_2^+	525.6		

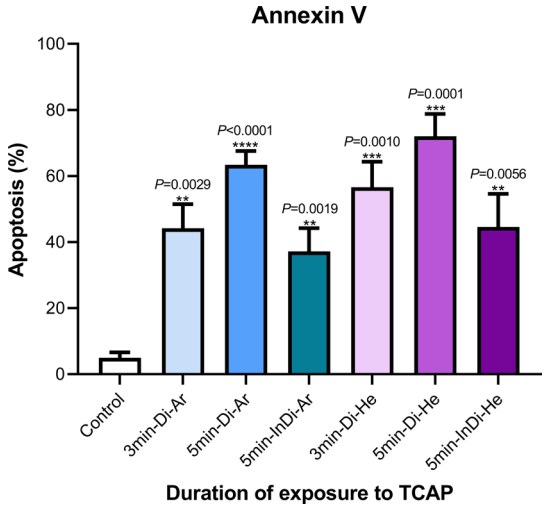

Figure 4. Evaluation of apoptosis induction in CCSCs through Annexin V. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

both gases, underscoring the safety and energy efficiency of the method. The voltages measured at the terminal of the secondary copper wire were 0.367 kV for argon and 0.304 kV for helium, which are significantly lower than the initial voltages applied, highlighting the efficiency of the transfer process. These findings are consistent with previous studies that suggest a reduction in plasma voltage is integral to minimizing energy consumption in transfer plasma applications.[76,77]

The production of RONSs during the TCAP treatment plays a crucial role in its anticancer efficacy.[78] The generation of species such as singlet oxygen (O) and nitric oxide (NO) has been shown to promote apoptosis in cancer cells by inducing oxidative stress and disrupting cellular homeostasis.[79] As illustrated in Figure 3, which pertains to optical emission spectroscopy, the TCAP process generates a wide variety of excited species, such as Ar I, Ar II, O I, O III, N I, N II, N III, O_2^+, and N_2^+ for argon and He I, O I, O II, N I, N II, N IV, and

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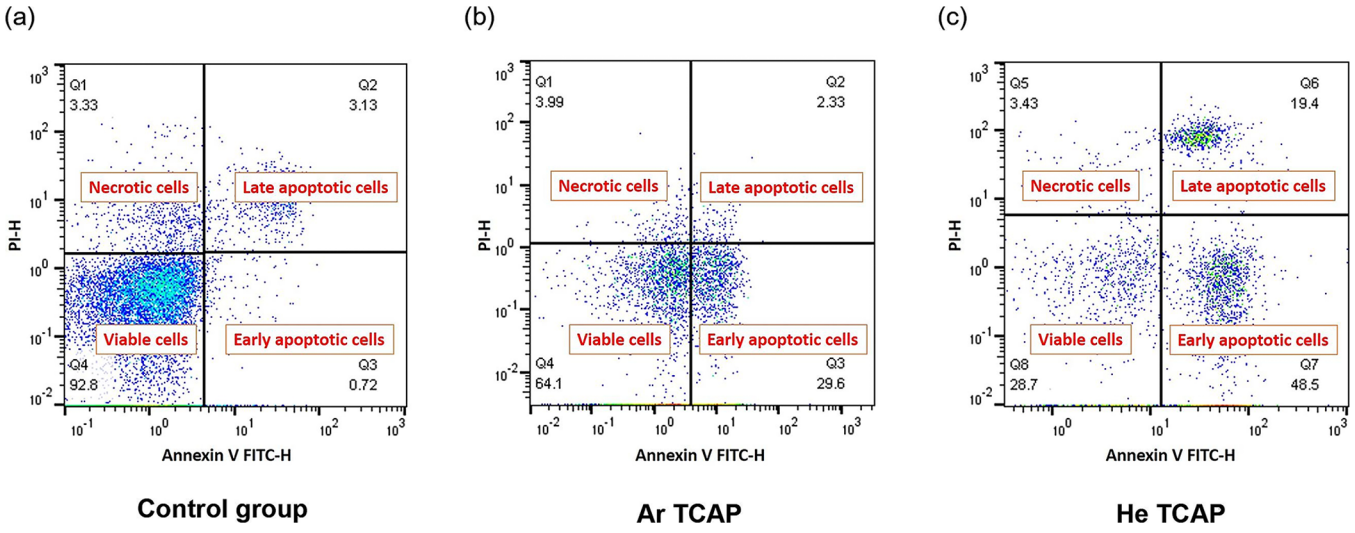

Figure 5. Annexin V and PI staining results for (a) the control group, (b) CCSCs treated with argon-based TCAP for 5 min, and (c) CCSCs treated with helium-based TCAP for 5 min. Flow cytometric analysis divided the cells into four quadrants: the fourth quadrant (Q4 and Q8) represents viable cells, the third quadrant (Q3 and Q7) corresponds to early apoptotic cells, the second quadrant (Q2 and Q6) indicates late apoptotic cells, and the first quadrant (Q1 and Q5) represents necrotic cells.

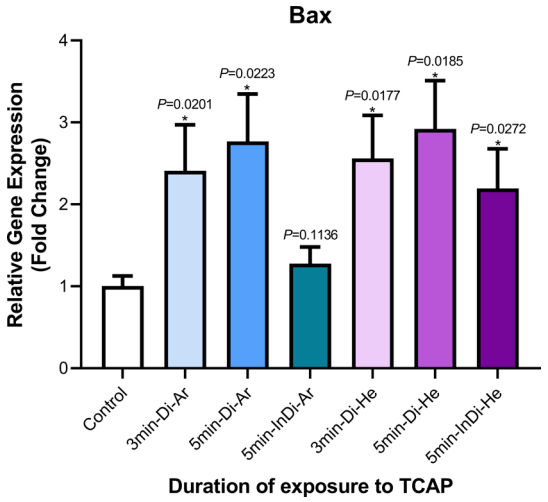

Figure 6. Bax gene expression levels in CCSCs following treatment with TCAP. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

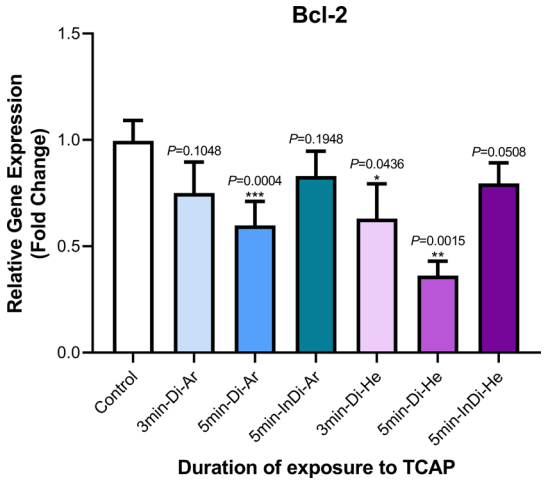

Figure 7. Bcl-2 gene expression levels in CCSCs following TCAP treatment. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and $*p < 0.05$.

N_2^+ for helium. Table 1 contains details about these species. These species are responsible for the cytotoxic effects of TCAP, as they trigger oxidative damage in CCSCs.[80] Previous studies have indicated that such reactive species can selectively induce apoptosis by interacting with cellular components such as lipids, proteins, and nucleic acids, leading to cell death.[81,82] The selective generation of these reactive species in TCAP suggests a tailored approach to cancer therapy, where only targeted cells are exposed to the harmful effects of RONS, thus sparing healthy tissues from collateral damage.[83,84]

In total, the following are TCAP's significant scientific innovations:

- Increased safety: the initial DBD voltage is reduced to safer levels at the end of the second copper wire, aligning the electrical impedance of the plasma with the body's natural resistance. This minimizes the risk of electrocution.
- Triple ionization: the TCAP method involves three stages of ionization—first within the DBD reactor, followed by ionization during each transfer step. This process enhances particle energy, velocity, and collision frequency, leading to increased production of RONSs. As a result, the plasma more effectively penetrates cancer cells, improving treatment efficacy.
- Energy efficiency: plasma generated in the DBD reactor is transmitted through copper wires. The copper wire structure localizes plasma generation to filaments, which reduces energy consumption compared to systems that generate plasma throughout the entire volume.

Biologically, apoptosis induction is a critical aspect of TCAP's anticancer mechanism,[85] as demonstrated by the Annexin V-FITC and flow cytometry analysis with Flomax software. As illustrated in Figure 4, the foregoing result is corroborated by the observation that TCAP-treated CCSCs, particularly those treated with direct helium gas, exhibit apoptotic triggering ($****P < 0.0001$).

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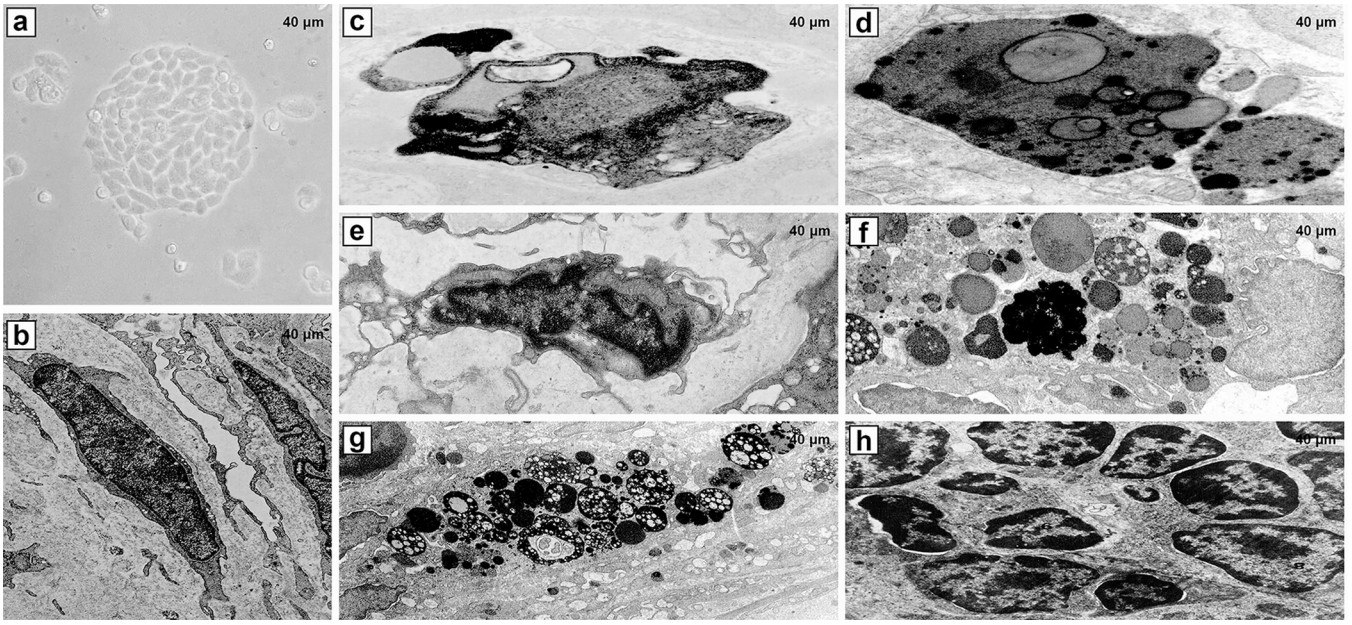

Figure 8. Transmission electron micrographs of CCSCs: (a) three-dimensional inverted microscopic image of CCSCs; (b) transmission electron microscopy (TEM) image of a representative colon cancer stem cell; (c) TEM image of CCSCs following 5 min of direct exposure to argon TCAP; (d) TEM image of CCSCs after 5 min of direct exposure to helium TCAP; (e) TEM image of CCSCs after 3 min of direct exposure to argon TCAP; (f) TEM image of CCSCs after 3 min of direct exposure to helium TCAP; (g) TEM image of CCSCs following 5 min of indirect exposure to argon TCAP; (h) TEM image of CCSCs after 5 min of indirect exposure to helium TCAP.

The percentages of live cells (Q4: Annexin V-FITC−/PI−), early apoptotic cells (Q3: Annexin V-FITC+/PI−), late apoptotic cells (Q2: Annexin V-FITC+/PI+), and necrotic cells (Q1: Annexin V-FITC−/PI+) were analyzed. As presented in Figure 5, the data indicated a significant increase in the percentages of early (Q3) and late (Q2) apoptotic cells in CCSCs treated with TCAP argon (Q3 = 29.6, Q2 = 2.33%) and TCAP helium (Q7 = 48.5, Q6 = 19.4%) compared to the control group (Q3 = 0.72, Q2 = 3.13%). This is consistent with prior research indicating that CAP treatment can enhance apoptosis in cancer cells through the generation of RONS.[86] The observed increase in apoptotic cells, especially in the helium-treated group, suggests that the type of gas used in TCAP plays a pivotal role in determining its effectiveness.

Subsequently, the expression of the genes Bax and Bcl-2, which are known to be standard indicators for apoptosis detection, denotes the transition to apoptosis.[87] Bax promotes mitochondrial membrane permeabilization, triggering apoptosis,[88] while Bcl-2 opposes this action, preventing cell death.[89] The balance between Bax and Bcl-2 is critical in determining cell fate.[90] This equilibrium regulates whether cells undergo apoptosis or survive in response to oxidative and other cellular stresses.[91] Figure 6 demonstrates that CCSCs exposed to TCAP exhibit significantly higher Bax protein levels compared to the control group (*p = 0.0177). Specifically, CCSCs treated with helium-based TCAP show a more pronounced increase in Bax expression than those treated with argon-based TCAP. Additionally, the direct TCAP application results in a greater increase in Bax levels compared to the indirect method.

Similarly, Figure 7 reveals a significant reduction in Bcl-2 levels relative to the control group (***p = 0.0004). This reduction is more prominent in helium TCAP-treated CCSCs than in those treated with argon TCAP and is also more substantial with the direct approach compared to the indirect method. In this regard, a notable rise in the ratio of Bax to Bcl-2 was observed, indicating the key to cell death. Notable is also the fact that Bcl-2 had a higher decrease rate than Bax. This differential impact might suggest that pro-survival rather than pro-apoptotic pathways are first disrupted more selectively by TCAP. Bcl-2 has a critical role in the survival of cancer cells; therefore, Bcl-2's substantial decrease may have therapeutic benefits, particularly for malignancies with high Bcl-2 expression.[92] Finally, it can be said that TCAP has a high potential for changing factors affecting cell death. In addition to apoptosis, TCAP's effects on CCSC morphology provide further evidence of its potential as an effective cancer therapy. Transmission electron microscopy (TEM) images in Figure 8 revealed significant ultrastructural changes in CCSCs exposed to TCAP. An inverted microscope was employed to capture a high-magnification 3D photomicrograph in Part (a), highlighting the spheroidal structure of colon cancer stem cell aggregations. As a reference for untreated cells, Part (b) depicts a typical colon cancer stem cell with a well-defined nucleus, organelles, and normal cell size. Part (c) illustrates the cellular effects following a 5 min direct exposure to Argon TCAP, revealing high chromatin density, chromatin margination, a large number of apoptotic bodies, and secondary lysosomes. Part (d) describes the effects of a 5 min direct exposure to helium TCAP on colon cancer stem cells, where apoptotic bodies, membrane blebbing, numerous secondary lysosomes, euchromatic nuclei, well-developed organelles such as the endoplasmic reticulum, and transparent mitochondria are visible. In Part (e), a 3 min direct exposure to Argon TCAP shows early apoptotic characteristics, such as increased chromatin margination and density. Part (f) demonstrates the cellular response after a 3 min direct helium TCAP exposure, showing high chromatin density, chromatin margination, apoptotic bodies, plasma membrane blebbing, and secondary lysosomes. Part (g) displays the results of a 5 min indirect exposure to Argon TCAP, showing apoptotic cells,

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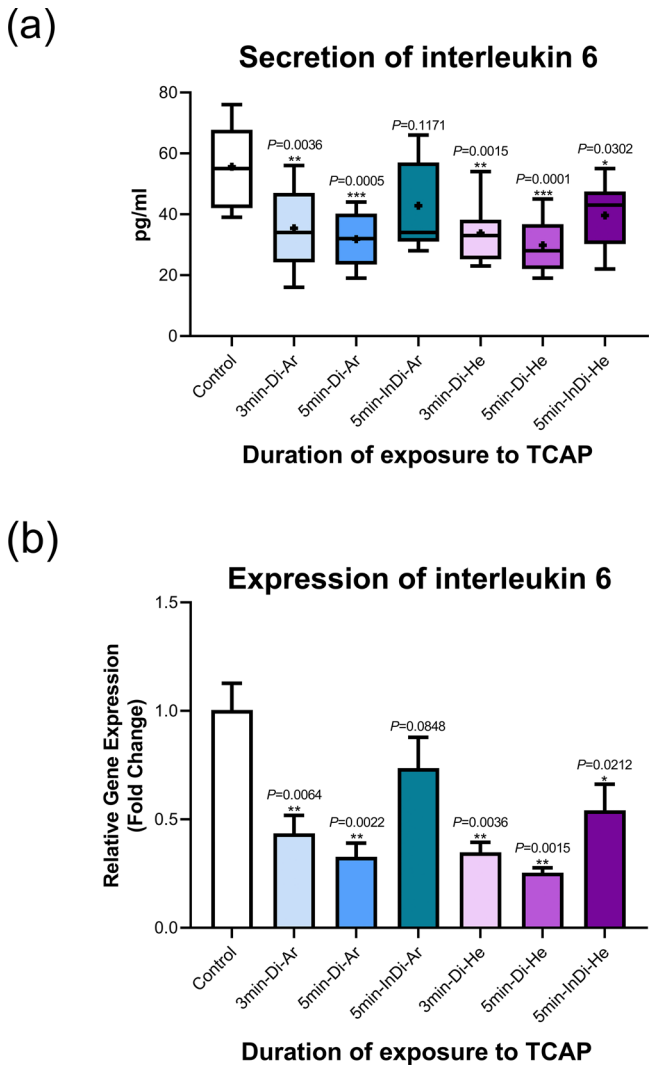

Figure 9. (a) Secretion and (b) expression of IL-6 related to CCSCs in the control and TCAP-treated groups. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and *p < 0.05, **p < 0.01, ***p < 0.001.

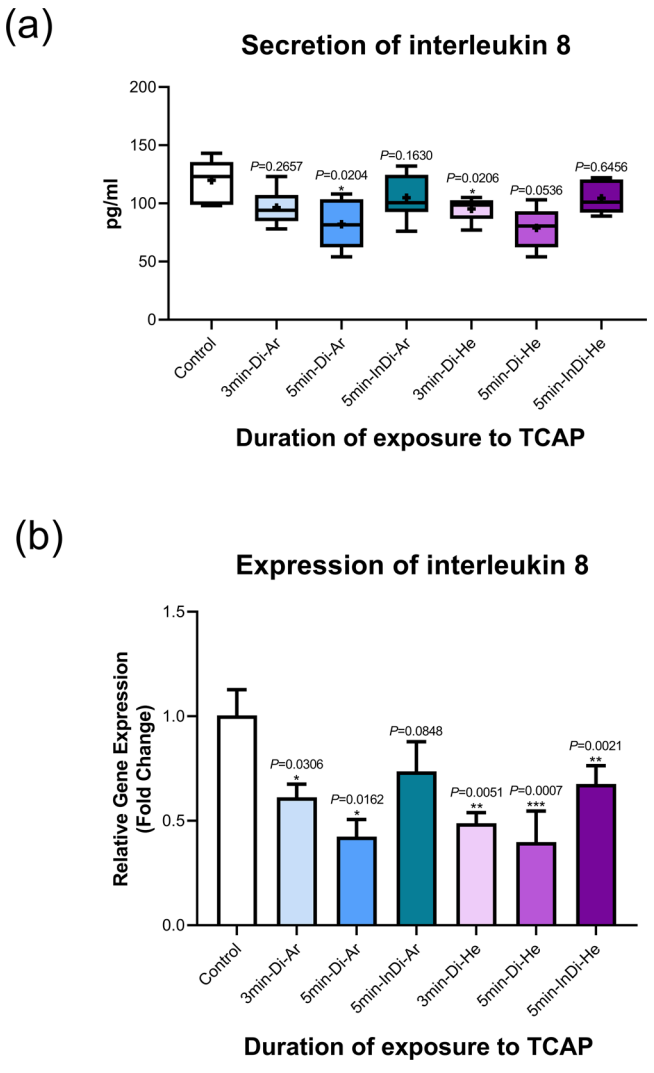

Figure 10. (a) Secretion and (b) expression of IL-8 related to CCSCs in the control and TCAP-treated groups. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and *p < 0.05, **p < 0.01, ***p < 0.001.

elevated chromatin density, cell blebbing, and numerous secondary lysosomes. Lastly, Part (h) outlines the effects of a 5 min indirect helium TCAP exposure, characterized by significant nuclear and chromatin disintegration, formation of intracellular vacuoles, and loss of intracellular organelles. These observations are characteristic features of apoptosis, confirming that TCAP induces cellular damage that leads to programmed cell death. The morphological alterations were more pronounced in cells treated with helium-based TCAP, suggesting that this gas may induce more severe cellular damage compared to argon. These findings are consistent with previous studies that have shown that CAP-induced apoptosis is associated with specific morphological changes in cells, including chromatin condensation, membrane blebbing, and the formation of apoptotic bodies.[93]

Inflammatory factors, particularly interleukin-6 (IL-6) and interleukin-8 (IL-8), play a crucial role in maintaining the stem-like characteristics and proliferative capacity of CCSCs.[94] In this study, TCAP treatment led to a significant reduction in the levels of IL-6 and IL-8. Based on the results, treated CCSCs release IL-6 and IL-8 in low to medium amounts, as seen in Figures 9 and 10. In treated CCSCs, the effect of various gases on this pattern is striking. When comparing TCAP ignited with helium gas to TCAP ignited with argon gas, the findings reveal a significant reduction in the expression of IL-6 and IL-8 (**p = 0.0015 for IL-6 and ***p = 0.0007 for IL-8). So, the treatment effect of TCAP of helium surpasses that of TCAP of argon. Furthermore, the direct TCAP treatment exhibited a more pronounced effect than the indirect treatment. Specifically, the reduction in pro-inflammatory cytokines IL-6 and IL-8 was significantly greater in the direct mode than in the indirect mode. This suggests that direct exposure to TCAP more effectively suppresses inflammatory signaling pathways, which may contribute to its enhanced ability to target and inhibit CCSCs. Given that CCSCs depend on an inflammatory milieu to preserve their stem-like characteristics and proliferation, a decrease in IL-6 and IL-8 is essential.[95,96] The reduction in these cytokines implies that TCAP interferes with proinflammatory signals that are vital to the survival of CCSCs, which may lessen the cells' resistance to therapy and the likelihood that the tumor would return. It is possible that mesenchymal stem cells' inhibition of inflamma-

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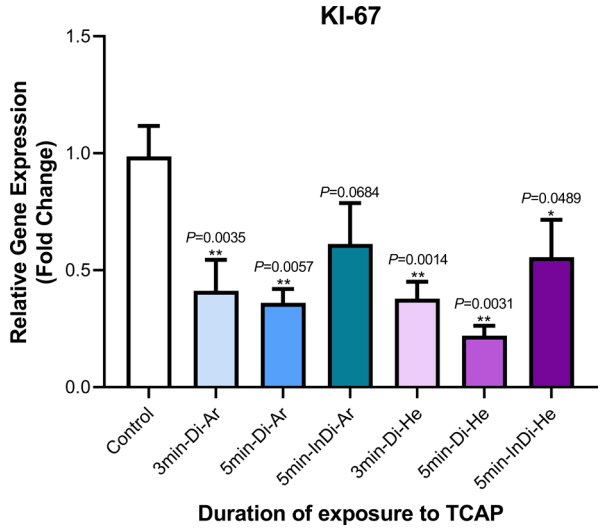

Figure 11. Levels of the proliferation-related factor KI-67 for CCSCs in control and TCAP-treated groups. Data are presented as mean + (SD), one-way ANOVA, Tukey test, and *p < 0.05, **p < 0.01.

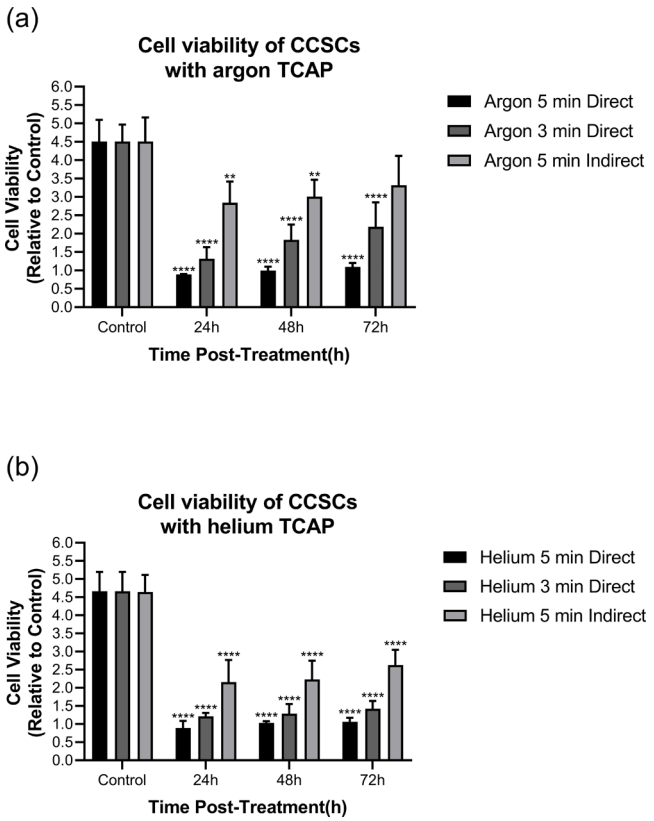

Figure 12. Assessment of CCSC viability via MTT assay after plasma treatment: (a) argon TCAP and (b) helium TCAP. Data are presented as mean + (SD), two-way ANOVA, Tukey test, and **p < 0.01, ****p < 0.0001.

tory signals prevents them from metastasizing and renewing tumors.[97] Additionally, TCAP may affect the immune system more broadly by reducing IL-6 and IL-8 levels. Through the recruitment of regulatory T cells and myeloid-derived suppressor cells, which inhibit antitumor immunity, these cytokines aid in tumor-associated immunosuppression.[98] TCAP may improve immune responses to cancer by lowering these cytokines, which would improve immune surveillance and make it possible to eradicate cancer cells more successfully.

The impact of TCAP on CCSC proliferation was further assessed by evaluating K_i-67 expression, a marker of cell proliferation. K_i-67 is a recognized marker for cell proliferation,[99] and its reduction following TCAP treatment suggests that TCAP can effectively inhibit the growth and division of CCSCs. As depicted in Figure 11, the evaluation of K_i-67 expression in CCSCs showed a significant decrease across all TCAP-treated groups compared to the untreated control (**p = 0.0014). Notably, the type of gas used played a critical role, with helium demonstrating greater effectiveness than argon. Additionally, the direct mode of TCAP application produced more pronounced effects than the indirect mode. This observation is particularly important, as CCSCs are known to resist conventional treatments such as chemotherapy and radiotherapy due to their self-renewal and proliferative capabilities.[100] By specifically targeting CCSC proliferation, TCAP offers a promising approach to overcoming the resistance mechanisms associated with these cells. The reduced K_i-67 expression may also suggest that TCAP induces a dormant state in CCSCs, potentially inhibiting their role in tumor growth or metastasis.[101] Alternatively, this decrease could indicate the activation of cell death pathways, as TCAP has been shown to promote apoptosis and necrosis in cancer cells through reactive oxygen species (ROS)-mediated mechanisms.[102]

Finally, the viability of CCSCs subjected to TCAP treatment was assessed using the MTT assay. According to Figure 12, the survival rate of CCSCs treated with TCAP is significantly reduced compared to the untreated cells (****p < 0.0001 for both argon and helium TCAP). Furthermore, the survival rate of CCSCs, when directly treated with TCAP of helium, is considerably lower than with TCAP of argon under the same conditions. This observation suggests that TCAP of helium exerts more pronounced effects than TCAP of argon. This reduction in survival may be attributed to the excessive production of intracellular ROS, leading to oxidative damage that exceeds CCSCs' repair capacity.[103] While CCSCs typically resist oxidative stress through enhanced antioxidant systems, our findings suggest that TCAP overcomes these defenses, resulting in a marked decrease in cell viability.[104] The mechanisms by which TCAP induces CCSC death likely involve ROS-induced damage to vital cellular components such as DNA, proteins, and lipids, triggering apoptosis, necrosis, or ferroptosis, depending on the severity and location of the damage.[105−107] These results align with prior research showing that TCAP causes mitochondrial dysfunction, releases proapoptotic factors, and activates cell death pathways in cancer stem cells.[108] Consequently, this developed method holds promise as an effective instrument for the therapy and control of colon cancer.

4. CONCLUSIONS

This study demonstrates how well the TCAP approach targets and triggers apoptosis in CCSCs. Through the use of transfer plasmas and a unique method incorporating argon and helium gases, TCAP shows a notable decrease in plasma voltage, increasing treatment safety and lowering energy usage. Important discoveries include a clear increase of apoptosis via Bcl-2 downregulation and Bax overexpression, which favors pathways involved in cell death. Furthermore, TCAP significantly decreased the viability of CCSCs and successfully

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decreased the levels of pro-inflammatory cytokines IL-6 and IL-8. It also altered the proliferation marker K_i-67. Significantly, helium-based TCAP had stronger anti-inflammatory and apoptotic effects than argon, with direct treatment modes outperforming indirect options. These results suggest that TCAP can selectively target and eliminate CCSCs via the generation of RONS, offering a potential strategy to overcome cancer resistance mechanisms. This novel plasma-based treatment holds significant promise for future cancer therapies, particularly for malignancies characterized by high Bcl-2 expression and inflammatory signaling.

■ ASSOCIATED CONTENT

Data Availability Statement

Upon acceptable request, the corresponding author will provide the study's data.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10378.

TCAP-produced reactive species (PDF)

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Author Contributions

The main draft was written by A.S. and L.R., who also conducted the research, gathered the data, and evaluated it. In addition, they examined and edited the essay, conceptualizing and designing the tests. T.M. contributed to the supervision and methods. S.Y.M. was involved in methodology. F.S. took part in gathering data. Every author provided feedback on the manuscript and exchanged views about the findings.

Notes

The authors declare no competing financial interest.

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