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REVIEW

PLASMA PROCESSES AND POLYMERS

On the selective killing of cold atmospheric plasma cancer treatment: Status and beyond

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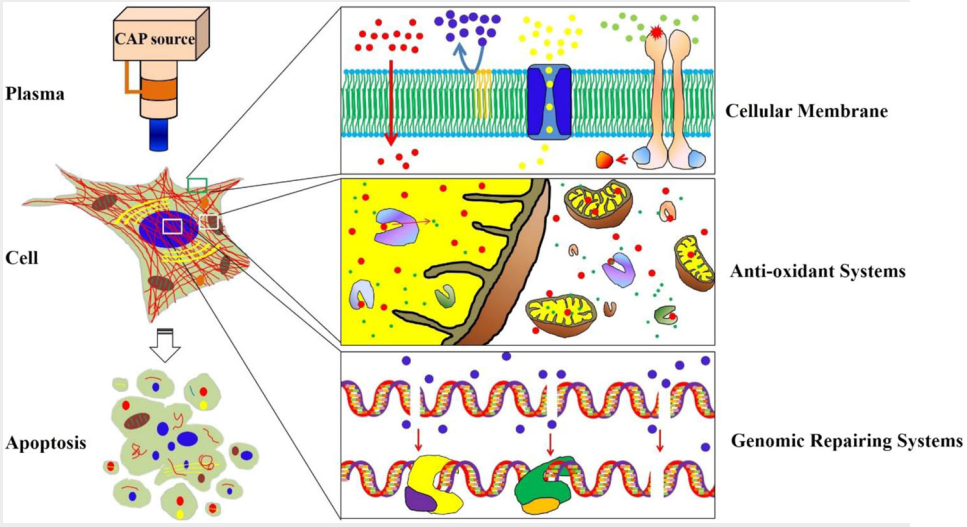
Abstract

Compared with many chemotherapy and radiotherapy modalities, selective killing of cancer cells is a pivotal feature of cold atmospheric plasma (CAP). Understanding its underlying mechanism will build the foundation of CAP-based cancer treatment. Additionally, we provided forward-looking thinking to extend the definition of selectivity from conventional cases involving a single-cell line to a coculture case. Finally, the newly established physically based treatment strategy provides unprecedented visions to realize selectivity beyond the previously established concepts based on reactive species and direct killing effect.



KEYWORDS
cold atmospheric plasma, cancer treatment, nonthermal plasma, selectivity

1 | INTRODUCTION

Cold atmospheric plasma (CAP) is an ionized gas with a near-room temperature, composed of complex multi-chemical and physical factors, has shown promising application in cancer treatment. Over the past decade, significant progress has been made in this direction. A typical helium CAP jet source is shown in Figure 1a. Due to the weak thermal effect, CAP can treat skin and tissue without causing thermal damage and other side effects.[1,2] CAP has shown promising potential as a novel anticancer modality over the past decade, particularly for

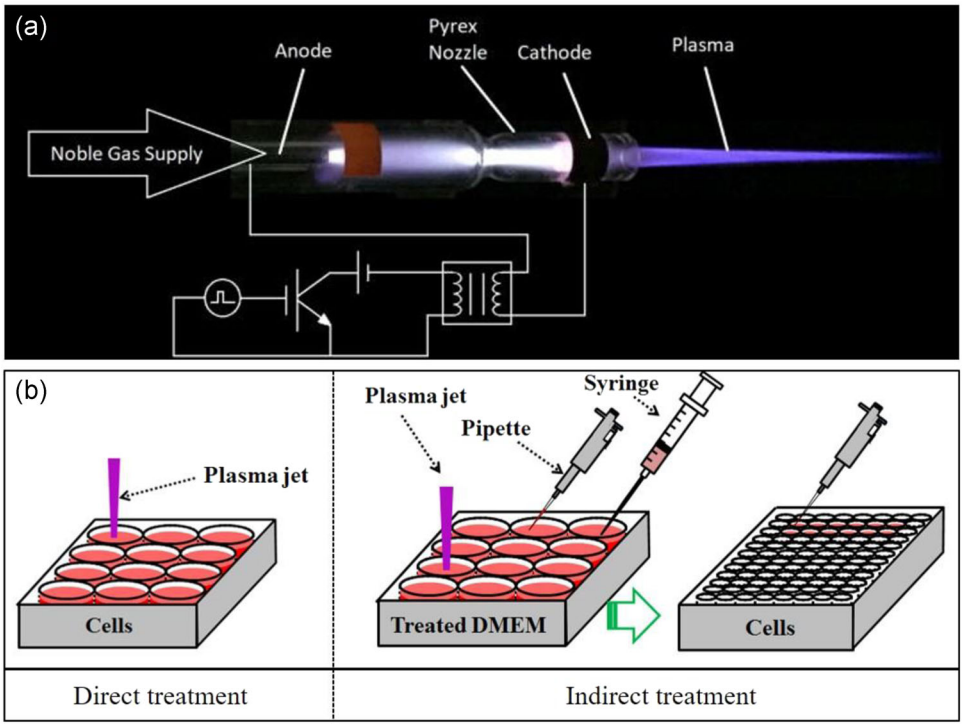


FIGURE 1 A typical CAP jet source and its use in cancer treatment. (a) A helium CAP jet. Reproduced with permission from Lin and Keidar[14] (b) The typical CAP treatment based on CAP jet. Reproduced with permission from Yan et al.[4] CAP, cold atmospheric plasma; DMEM, Dulbecco's modified Eagle's medium

TABLE 1 Some typical selective anticancer demonstrations in vitro

Cancer cell lines	Normal cell lines	References
Breast cancer (MCF7)	Breast (M5655)	[19]
Benign prostatic hyperplasia (BPH-1)	Prostate primary epithelial cells	[20]
Colorectal cancer (HCT116)	Colon epithelial (FHC)	[21]
Cervical cancer (HeLa)	Fibroblast (HFB)	[22]
Gastric cancer (SC-2-NU)	Human fibroblast (WI-38)	[23]
Glioblastoma (U251SP)	Astrocytes (ACBRI-371)	[24]
Head and neck squamous carcinoma (JHU-022)	Oral cavity epithelial (OKF6)	[25]
Hepatoma cancer (HepG2)	Liver (L-02)	[26]
Lymphoma (U937)	Blood primary monocyte cells	[27]
Lung cancer (H460)	Lung fibroblast (L132)	[28]
Ovarian clear carcinoma (TOV21G)	Peritoneal mesothelial (OHFC)	[29]
Squamous cell carcinoma (PAM212)	Wild-type keratinocytes (WTK)	[30]

the in vitro treatments performed on either traditional cell culture or 2D/3D cell culture using spheroids or scaffolds.[3–7] A simple treatment above the subcutaneous tumor site could extend the life of mice by a strong inhibition effect on tumor size, which is a non-invasive nature of CAP.[8–10] Besides, CAP may also cause immunogenic cell death in vivo, which is different from the direct CAP-caused cell death focused in this review.[11]

TABLE 2 Lifetimes of some reactive species

Reactive species	Half-life	References
Hydroxyl radical (•OH)	~ 10^{-9} s (in vivo)	[41]
Hydroxyl radical (•OH)	~ 10^{-9} s (in vitro)	[42]
Superoxide radical ($O_2^{\bullet -}$)	~ 10^{-6} s (in vivo)	[41]
Superoxide radical ($O_2^{\bullet -}$)	~ 10^{-3} s (in vitro)	[43]
Hydrogen peroxide (H_2O_2)	~ 10^{-5} s (in vivo)	[41]
Hydrogen peroxide (H_2O_2)	Hours, days, months, or longer (in vitro)	[44]
Singlet oxygen ($^1O_2$)	~ 10^{-6} s (in vivo)	[39]
Singlet oxygen ($^1O_2$)	~ 10^{-6} s (in vitro)	[40]
Nitric oxide radical ($NO^{\bullet}$)	~ 1 s (in vivo)	[41]
Nitric oxide radical ($NO^{\bullet}$)	Seconds, minutes, hours (in vitro)	[45]
Peroxynitrite ($ONOO^-$)	~ 1 s (in vivo)	[41]
Peroxynitrite ($ONOO^-$)	~ 10^{-3} s (in vitro)	[46]
Nitrite (NO_2^-)	Days, months, or longer (in vitro)	[47]
Nitrate (NO_3^-)	Days, months, or longer (in vitro)	[48]

Cancer therapy is a big challenge for modern medicine. Many chemotherapy and radiotherapy modalities generate side effects or non-ignorable harm to patients.[12,13] It is urgent to find a novel selective anticancer modality, which will have an attractive application perspective. One attractive feature of CAP is its selective anticancer capacity demonstrated in many cases.

The widely acknowledged concept of selectivity of CAP treatment means that either direct CAP treatment or the indirect CAP treatment based on the CAP-treated medium can selectively cause strong cell death in cancer cell lines while only causing slight damage in normal cell lines under the same experimental conditions (Figure 1b).[15] It is necessary to point out that all these tests were performed by treating a single cancer cell line or normal cell line in monoculture. It was initially reported by Georgescu and Lupu,[16] Kim et al.,[17] and Zirnheld et al.[18] in their works about murine melanoma cells and human colon cancer cells in 2010. A summary of some typical selective cases is presented in Table 1. Such a widely observed feature suggests some common features or responses of cancer cells and normal cells to CAP treatment.

2 | GENERAL ANTICANCER MECHANISM IN VITRO

The typical CAP-originated reactive oxygen species (ROS) include singlet oxygen ($^1O_2$), hydroxyl radical (•OH), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2).[31] The typical CAP-originated reactive nitrogen species (RNS) includes peroxynitrite ($ONOO^-$), nitrite (NO_2^-), nitrate (NO_3^-), and nitric oxide (NO).[32,33] Many reactive species from CAP are toxic to cancer cells. OH, a typical short-lived reactive species, is highly reactive to many vital molecules, including DNA, RNA, proteins, and phospholipid.[34] $ONOO^-$ can damage the function of antioxidant enzymes and other molecules.[35,36] H_2O_2, a typical long-lived reactive species, is toxic to various cancer cell lines.[37] For instance, H_2O_2 can cause oxidation on many proteins and DNA.[38]

The main chemical components in CAP can be divided into two categories: short-lived and long-lived reactive species. The sharp difference in reactive species' lifetime length determines their availability and therefore their ability to impact biological processes (Table 2). For short-lived reactive species, such as $O_2^{\bullet -}$, $OH^{\bullet}$, $^1O_2$, and $ONOO^-$, they can affect the cells just near the CAP source over a short-time length scale (nano-, micro-, and milliseconds).[39–41] Long-lived reactive species (NO_2^-, NO_3^-, O_3, NO, H_2O_2) can affect cells relatively far from CAP source through the diffusion in the layers of medium or solution over a long-time length scale. Such a difference will be much more apparent when comparing the different biological effects of direct CAP treatment with the indirect CAP treatment based on the CAP-treated medium or solution.

Generally, the anticancer mechanism in vitro is based on the biological effect of chemical factors in CAP, particularly ROS such as H_2O_2. In many cases involving direct CAP treatment on cells, RNS or ROS touched a medium layer or solution layer before they further affect cells. Such an interaction style is due to the widely used

in vitro experimental designs, where cells remain in culture conditions during treatment.[49,50] Similarly, in the case of using the CAP-treated medium or solution to affect cells, the long-lived reactive species or other reaction products play a dominant role in determining cells' final fate.[24,51] Evidence for such is seen when the cell culture medium present during treatment is immediately exchanged for a fresh culture medium, completely inhibiting CAP treatment's cytotoxicity.[52] The presence of extracellular ROS scavengers, such as cysteine, pyruvate, and nordihydroguaiaretic acid (NDGA), can completely counteract CAP's cytotoxicity on cancer cells.[53,54]

The rise of intracellular ROS has been widely reported in many CAP-treated cancer cell lines.[54,55] CAP treatment cannot kill cancer cells once these cells have been pretreated with intracellular ROS scavengers, such as *N*-acetyl-cysteine (NAC), rotenone, D-mannitol, Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), diphenyleneiodonium (DPI), as well as apocynin.[11,54–63] Thus, the intracellular ROS rise has been regarded as the key mechanism to cause final cell death. The chemical essence of intracellular ROS rise is still mostly unknown because, in most cases, ROS assays are based on an oxidant-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), which is not a specific ROS probe.[21,64] H_2O_2, O_2^-, $OH^•$, and $ONOO^-$ may mainly contribute to the increased intracellular ROS.[27,55,65–67] ROS rise may be either due to (1) the diffusion of extracellular ROS or (2) the intracellular reactions and releases triggered by exogenous reactive species. For the first case, because most CAP-generated reactive species are polar or charged molecules, the transmembrane diffusion of ROS/RNS requires specific membrane proteins. However, molecular dynamics (MD) simulation recently found that transient pores (~15 Å) could be naturally formed on phospholipid bilayer (PLB) once all phospholipids are oxidized (Figure 2).[68] Thus, CAP's oxidative stress may indirectly form many transient pores on the cellular membrane, providing a fast routine for reactive species to enter the cytosol. RNS (NO, NO_2, N_2O_4) and O_3 may permeate more easily through the oxidized PLB than hydrophilic ROS, such as OH, HO_2, and H_2O_2.[69] Membrane consisting of combined nitro-oxidized lipid products may protect the transient pore formation in the presence of oxidized lipids.[70]

For the second case, complex intracellular pathways may be involved after CAP treatment. One pathway may overturn the redox balance by compromising on the intracellular antioxidant system. Naturally, the intracellular antioxidant system will resist intracellular ROS rise. Such a system includes small molecules, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH), as well as numerous enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and peroxiredoxin. The decreased ratio of GSH/GSSG and NADPH/$NADP^+$ has been observed in many CAP-treated cancer cell lines.[71] Similarly, the decreased expression of SOD and CAT in CAP-treated cancer cell lines has also been reported.[72] The intracellular ROS rise causes considerable cellular damage, such as mitochondria damage, DNA damage, cellular membrane damage, and endoplasmic reticulum

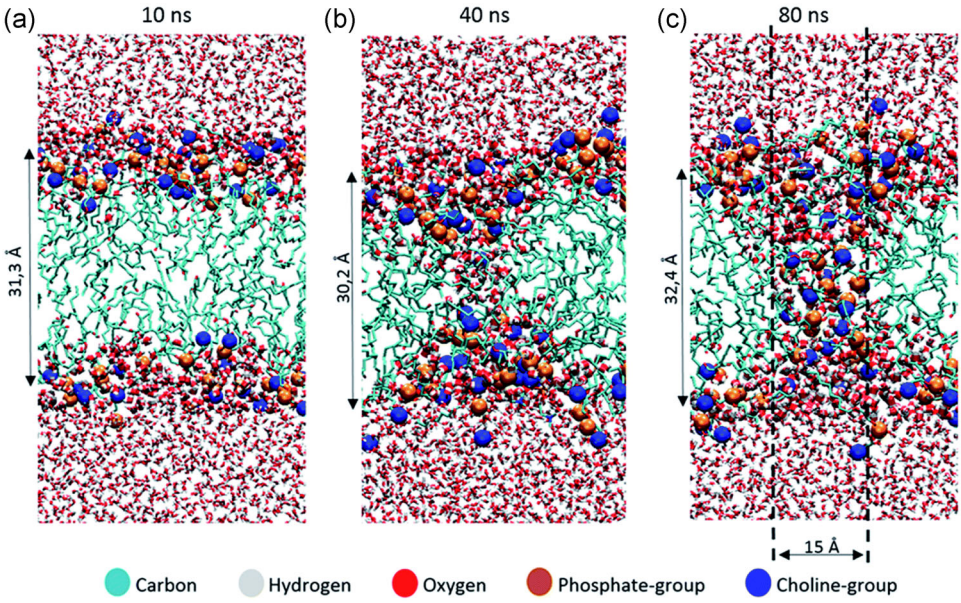


FIGURE 2 Snapshots of transient pore formation on phospholipid bilayer based on molecular dynamics simulation, after 10, 40, and 80 ns in the model system with 100% oxidation. With the permission of Van der Paal et al.[68]

damage, ultimately followed by cell death with a CAP dose-dependent trend.[28,73,74] For the cell death triggered by chemical factors, apoptosis is the primary cell death form, though necrosis and autophagy-associated cell death have also been found.[15,75–78]

Though CAP treatment's chemical essence is still disputable, ROS has been regarded as the keystone component to ultimately achieve cell death[4] and H_2O_2 may be a critical ROS in many cases.[27,28,44,51,65,67,79–81] Extracellular H_2O_2 scavengers, such as sodium pyruvate and cysteine, and cysteine derivatives, such as NAC, and CAT can strongly counteract the cytotoxicity of a CAP treatment. Other ROS, such as $O_2^{\bullet-}$, $OH^{\bullet}$, and $^1O_2$, may also contribute to cell death observed in some ROS-resistant cell lines' cell death, and $^1O_2$ has been regarded as a necessary killing factor in some studies.[61,82] RNS, such as NO_2^-, NO_3^-, and $ONOO^-$, may also contribute to CAP's cytotoxicity. For some cell lines, compared with a single H_2O_2 treatment, synergistically using H_2O_2/NO_2^- or $H_2O_2/NO_2^-/NO_3^-$ mixture can produce cytotoxicity, which is more close to the cytotoxicity due to CAP treatment.[83,84] Besides, $ONOO^-$ may contribute to kill myeloma cells by a nanosecond pulsed N_2/O_2 plasma.[85]

3 | SELECTIVE ANTICANCER MECHANISM

Under the same experimental conditions, a CAP treatment can kill cancer cells much more than normal counterpart cells in many studies.[16,17,86] It has been suggested that the selective death of cancer cells may be mainly due to selective apoptosis after CAP treatment.[4,16,17,86] Due to the robust metabolism rate in cancer cells, the basal ROS level is naturally higher in cancer cells than normal cells.[87–89] When additional ROS stress is exerted, allowing them to be killed more easily than normal cells (Figure 3).[90,91] However, the basal ROS level has been difficult to determine accurately by assays, such as fluorescent ROS assays.[54] The selective rise of intracellular ROS in cancer cells is the most widely observed cellular change following CAP treatment, a deeper understanding of which could be central to understanding CAP-based anticancer selectivity.[21,54,64,71,92]

It is necessary to point out that H_2O_2 alone in a relatively high concentration or as the mediator of a series of anticancer drugs can selectively induce apoptosis in some cancer cell lines.[37,93,94] However, the observable selectivity cannot be simply explained by H_2O_2 alone. Selectivity may not be explained or exactly predicted by just one factor. On the one hand, CAP has complex chemical and physical components. Here, we only focused on a few ROS, such as H_2O_2 and $^1O_2$, and their potential role in selectivity. Other ROS, RNS, and physical factors may also contribute to the final cellular responses. On the other hand, even a single cell is an extremely complex system. Many proteins or other molecules may have different expression patterns in cancer cells and normal cells, many of which could potentially contribute to selectivity (Figure 4).

It is necessary to point out that any models or explanations need to be self-consistent with the widely observed experimental data or the acknowledged conclusions in plasma medicine. More importantly, the new models need to have universality, at least partial universality, because of the universality of selectivity. Besides, selectivity also exists when the CAP-activated

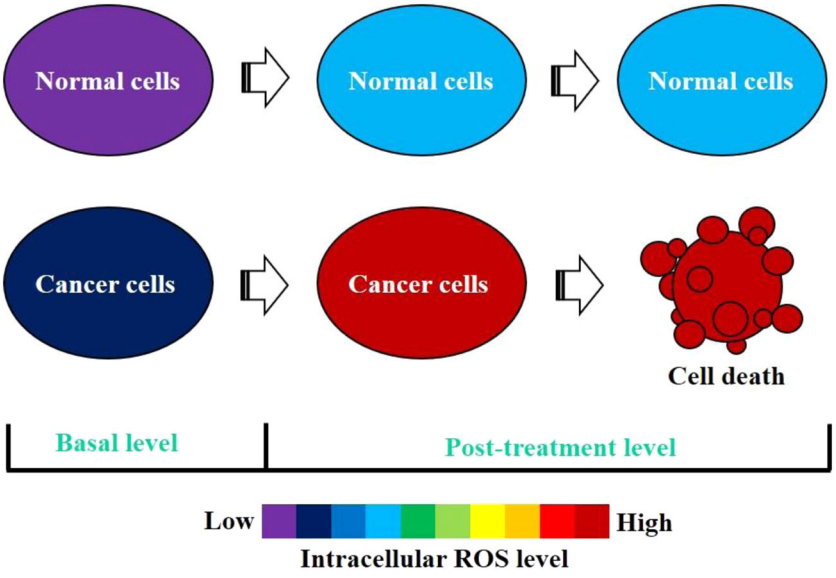


FIGURE 3 A schematic illustration of selective reactive oxygen species (ROS) rise in cancer cells. The selective ROS rise in the cold atmospheric plasma-treated cancer cells may be the pivotal clue to understand selectivity

solutions were used to affect many cancer cell lines.[24,59,60,83,95–98] Thus, the short-lived reactive species should not be considered in the selectivity caused by the CAP-activated solution or medium.

The candidate should be related to some general features that are common to many cancer cells, which, in turn, would be less common to their normal counterparts. Over the past 5 years, the roles of some factors have been proposed to explain selectivity. These efforts focus on different cellular features and provide unique visions and clues toward the final understanding of selective anticancer capacity based on CAP. Despite lacking experimental evidence, some related preliminary studies and simulation studies support these suggestions.

3.1 | Aquaporins (AQPs)

A model based on the different expression levels of AQP in cancer cells and normal cells has been proposed.[15] AQP facilitates the transmembrane diffusion of some small molecules, including H_2O, CO_2, NO, and NH_3.[99,100] Many studies confirmed that AQP (1, 3, 8, 9) also facilitates the passive transport of H_2O_2 across the cellular membrane in bacteria, plant, and mammalian cells.[101–104] The uptake of H_2O_2 in human colon adenocarcinoma cells (HT29) and cervical cancer cells (HeLa) was drastically enhanced via the expression of AQP 3.[103] MD simulation demonstrated that the free energy barrier of H_2O_2 across AQP 1 was lower than H_2O_2 across a palmitoyl-oleoyl-phosphatidylcholine PLB, suggesting that the diffusion of H_2O_2 into cells can be through AQP.[105] For yeast cells, the expression of AQP 8 caused strong cytotoxicity in 0.2 mM H_2O_2 solution. In contrast, even a 1.0 mM H_2O_2 solution could not cause noticeable death on the yeast cells without the expression of AQP 8 (Figure 5).[104] These studies demonstrated that the expression level of AQP could cause different cell death rates upon the same H_2O_2 treatment.

Human tissues have specific AQP expression patterns. For instance, AQP 4 is the main AQP expressed in the eyes, nose, and heart. AQP 1, 8, and 9 are the main AQP expressed in the liver. It is necessary to point out that many mammalian tissues express AQP 1, or 3, or 8, including the eyes, brain, lung, heart, liver, spleen, pancreas, kidney, ovary, and muscle.[106] These tissues were involved in many CAP-based cancer treatments in vivo. AQP plays pivotal roles in tumor-associated edema, angiogenesis, tumor proliferation, and migration. Many tumorous tissues, such as breast cancer, lung cancer, astrocytoma, and cervical cancer, express more AQP 1, 3, or 8 than healthy tissues.[107]

We hypothesized that different ROS transmembrane diffusion capacities between cancer cells and normal cells might contribute to the selective rise of intracellular ROS in cancer after CAP treatment. Many cancer cells express more AQP than their normal cells counterparts to sustain their vigorous proliferation and metabolism.

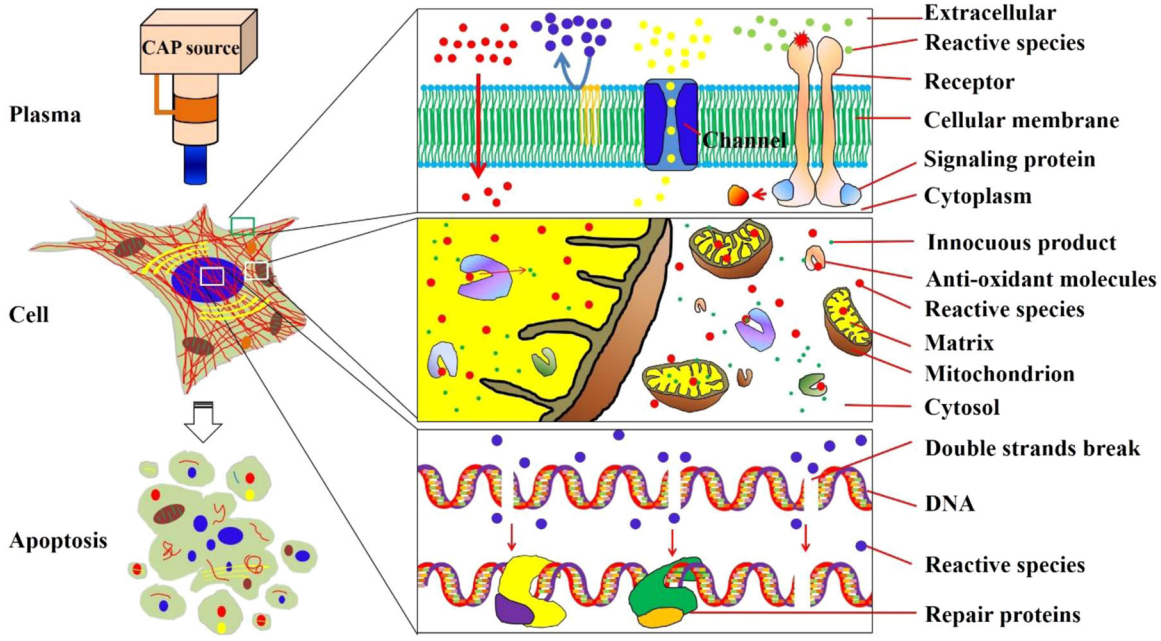


FIGURE 4 Multiple factors may contribute to selectivity by their specific expression in cancer cells and normal cells. Generally, it may include the entrance of reactive species into the cytosol, the impact of reactive species on cellular membrane, intracellular antioxidant system, and genome repairing system. Reproduced with permission from Yan et al.[15]

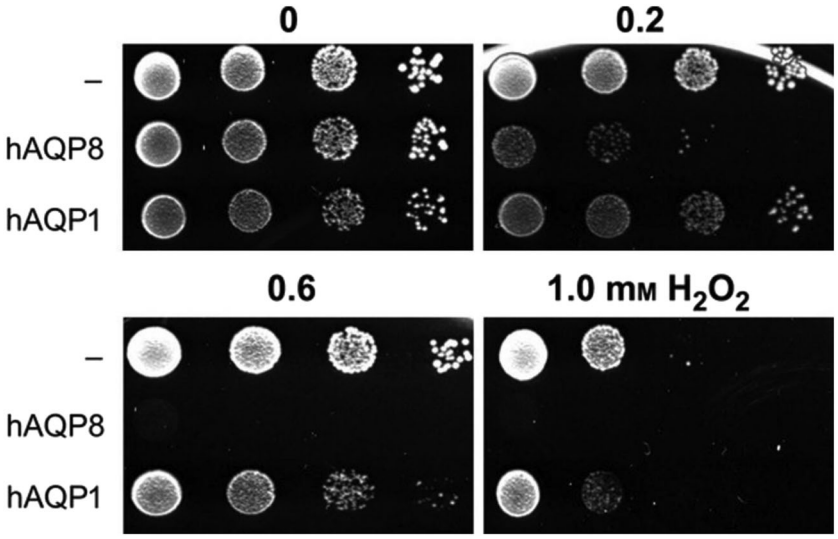


FIGURE 5 Expression of aquaporins (AQPs) affects H_2O_2 cytotoxicity on yeast cells. Yeast cells' phenotypic growth assay was performed on H_2O_2-rich agar media. Human orthodox AQP8 (hAQP8) and AQP1 (hAQP1) were expressed in some yeast cells. Cell density in each panel decreases from left to right. Reproduced with permission from Almasalmeh et al.[104]

Considering H_2O_2 generated in CAP treatment alone, the faster uptake of H_2O_2 by cancer cells will be faster than normal cells, contributing to the selective ROS rise and death in cancer cells. Though the direct experimental evidence is still lacking, some discussions can be given based on references and a preliminary study. The role of AQP in the cytotoxicity of CAP treatment has been explored in a glioblastoma cell line U87MG. AQP blockers, such as silver (Ag) atoms, can inhibit CAP's cytotoxicity on U87MG cells.[108] U87MG cells have a clear expression of AQP 1, 3, 8, and 9.[108] Silencing the expression of AQP 8 by microRNA significantly compromised treatment efficacy.[108] Silencing AQP 9 did not change the CAP treatment's cytotoxicity on U87MG cells. Thus, the specific role of the AQP family in CAP's anticancer capacity is still far from clear. A specific AQP may dominate the transportation of reactive species and further determine the cellular response to CAP. Further systematic and comparative studies on the full AQP family of cancer or normal cell lines are urgent.

3.2 | Antioxidant systems

The complete intracellular antioxidant systems in the cytoplasm and organelles include small antioxidant molecules, such as NAD(P)H and glutathione, and ROS-scavenging enzymes, such as CAT, SOD, peroxiredoxin, and glutathione peroxidases/reductases.[109] Among them, peroxiredoxin is the main target of H_2O_2 in mitochondria.[110] The redox balance between the uptake of exogenous ROS and intracellular ROS scavengers based on the intracellular antioxidant system may directly determine the intracellular ROS level.[102] A robust intracellular antioxidant system may weaken the cytotoxicity of CAP. For example, decreased/inhibited expression of Mn, Cu, and Zn-SOD increased the cell death of CAP-treated HeLa cells.[59] Overexpression of CAT also decreased the death rate of CAP-treated HeLa cells.[59] The expression of antioxidant enzymes in many cancer cells is less compared with normal cells.[87] For instance, CAT expression in cancer cells is less than normal cells in many cases.[111–113] Recently, it is found that stable intracellular glutathione levels only occurred in the CAP-treated ROS-resistant cell lines, which may be due to more expression of cysteine–glutamate antiporter xCT (SLC) in these ROS-resistant cell lines compared with other ROS-sensitive cell lines (Figure 6).[114] Generally, if normal cells demonstrate a more robust antioxidant system, they may be more resistant to exogenous ROS cytotoxicity, leaving them undamaged by CAP treatment.

There is an inversely proportional correlation between the H_2O_2 consumption rate of cancer cells and CAP treatment's cytotoxicity. For 10 cancer cell lines, the cancer cell line with a faster H_2O_2 consumption rate will be more resistant to CAP treatment. For example, melanoma cell line B16F10 can clear all extracellular H_2O_2 with the quickest speed, just <1 h after CAP treatment. Correspondingly, B16F10 cells show extreme resistance to CAP treatment. In contrast, bone osteosarcoma cell line U-2 OS shows the slowest extracellular H_2O_2 scavenging speed but owns the strongest vulnerability to CAP treatment's cytotoxicity (Figure 7a). This quasi-inversely proportional correlation strongly suggests the pivotal role of cancer cells' antioxidant system in CAP treatment's cytotoxicity.

Additionally, another general trend was that the cells, including cancer cells and normal cells expressing tumor suppressor p53, would be more resistant to CAP treatment than the cells without p53 or inhibited

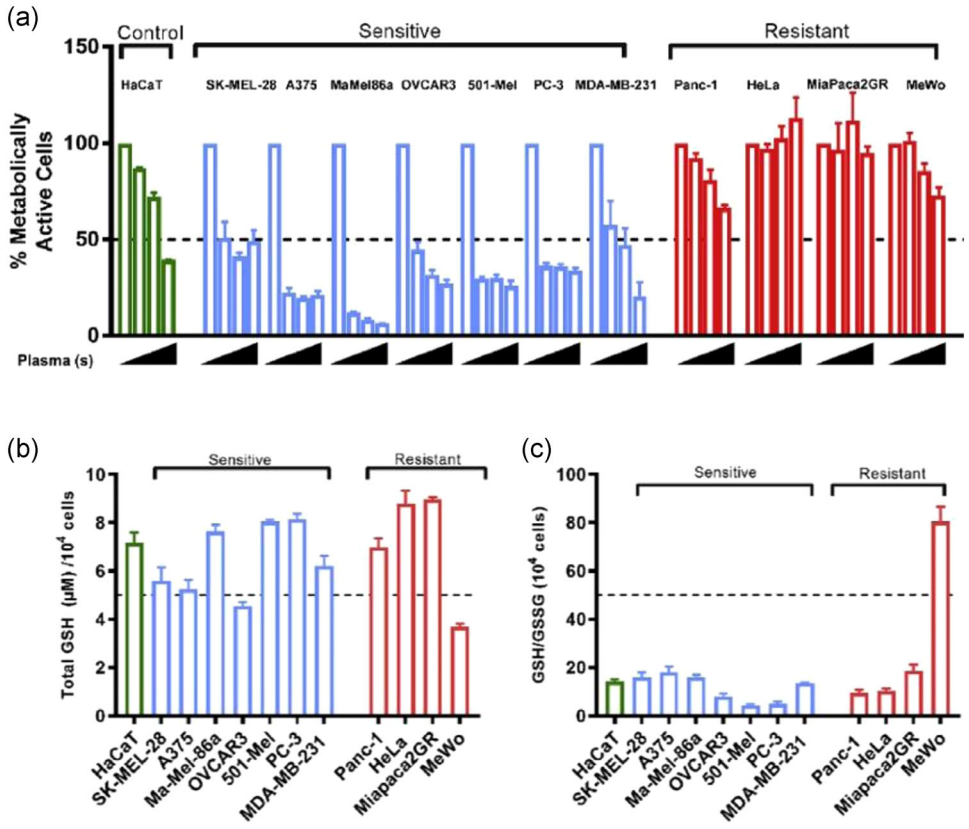


FIGURE 6 Sensitivity of cancer cell lines to cold atmospheric plasma (CAP) treatment. (a) Metabolic activity at 24 h after CAP treatment (0, 30, 60, 120 s). Cell lines that showed >50% reduction in metabolic activity after the 30 s of CAP treatment were categorized as "sensitive," and <50% reduction were categorized as "resistant" cell lines. (b) Basal glutathione (GSH) levels. (c) GSH/GSSG ratio. Reproduced with permission from Bekeschus et al.[114]

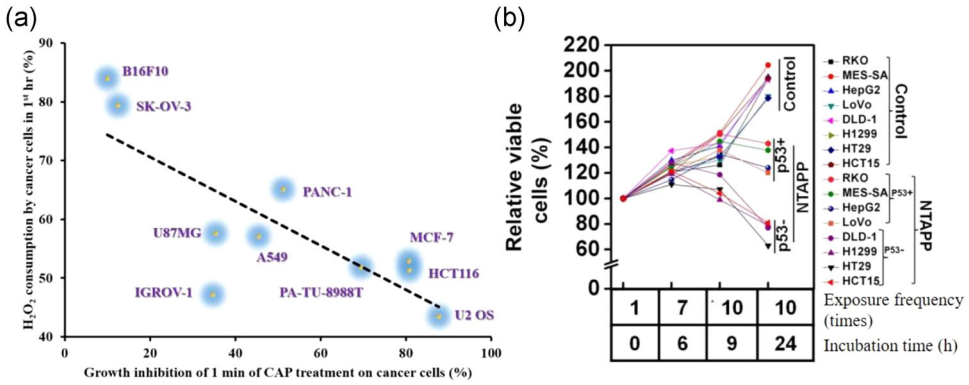


FIGURE 7 Two general trends in cancer treatment. (a) The extracellular H_2O_2 consumption rate of a cancer cell line is inversely proportional to cold atmospheric plasma (CAP) treatment's anticancer effect. The data were calculated based on the methods in Ref. [115]. Reproduced with permission from Yan et al.[115] (b) Highly preferential anticancer efficacy of CAP on cancer cells without functional p53. Both p53-proficient cell lines (RKO, MESSA, HepG2, G361, LoVo) and p53-deficient cell lines (DLD-1, H1299, HT29, HCT115) are cancer cell lines. "NTAPP" means CAP here. Reproduced with permission from Ma et al.[116]

expression of p53 gene (Figure 7b).[116] Loss of p53 gene is a pivotal step in the tumorigenesis of many tumors. Many tumors in the high tumorigenic stage lack p53 expression.[117] As a multifunction transcription factor, p53 protects the genome from ROS-caused oxidation by regulating many antioxidant enzymes' expression.[118–121] p53 gene is an important regulator of intracellular ROS levels.[122] Thus, the cancer cells lacking p53 gene may have a weaker antioxidant system, which explains stronger cell death after CAP treatment.

3.3 | Transient membrane pores

The transient membrane pore formation in the cellular membrane, particularly in the cytoplasm membrane, provides another perspective for understanding selectivity. Based on the different trends to form transient membrane pores on the cytoplasmic membrane after CAP treatment, the studies based on MD simulations provide new clues. The CAP-originated ROS can oxidize the lipids in the PLB of the cellular membrane. MD simulation demonstrates that such liquid oxidation may increase PLB's permeability to ROS in the extracellular space by decreasing the electric field threshold for transient pore formation.[123]

More importantly, MD simulation suggests the unique role of cholesterol in the permeation of reactive species across the PLB. It is found that the increased cholesterol fraction in the PLB will increase the PLB order and the transfer free energy barrier height and width, which results in a local free energy minimum at the center of the membrane to create extra free energy barriers (Figure 8).[68,124] Cholesterol may inhibit the pore formation on the cytoplasmic membrane.[68,124] Cholesterol may have specific distribution in cancer cells or normal cells. For example, compared with lymphocytes, leukemic cells have a lower cholesterol/phospholipid ratio.[125] It is reasonable to suggest that the CAP-originated reactive species may diffuse into cancer cells more quickly than normal cells in terms of the probability of forming transient pores on the cytoplasmic membrane. However, this is hinged whether the cholesterol/phospholipid ratio in cancer cells is commonly lower than normal cells because selectivity has been commonly observed in many cancer types. This point requires more experimental evidence to support it. So far, the available evidence may be just limited to lymphocyte and lymphoma cell types.

3.4 | Singlet oxygen

CAP also generates $^1O_2$, which may have a significant impact on cancer cells.[126,127] $^1O_2$ can inactivate membrane-bound CAT, inducing the generation of cancer cell-derived secondary $^1O_2$ and initiating mechanisms of ROS-/RNS-dependent apoptotic pathways.[128] Unlike other ROS/RNS, $^1O_2$ signaling may result in self-perpetuating apoptotic signaling from cell-to-cell and finally reach the tumor tissue in depth.[82] CAP may selectively work against cancer cells in vitro and even tumors in vivo based on CAP-derived $^1O_2$ and the different expression patterns of cytoplasmic membrane-localized CAT.[82] As shown in Figure 9, $^1O_2$ from CAP initially only reaches a tumor's surface and inactivates some membrane-associated CAT. Later, at the local CAT inactivation site, secondary $^1O_2$ is generated by cancer cells, which inactivates more other CAT on the same cells and neighboring cells. The generation of secondary $^1O_2$ and CAT inactivation spread into deeper tissue. Finally, apoptotic cells appear in the upper layers of tissue when the generation of secondary $^1O_2$, CAT inactivation, and RONS signaling spread into deeper tissue.

To date, it is urgent to have more evidence to demonstrate that the robust membrane-bound CAT expression is specific on cancer cells rather than on normal cells, which clearly will be a key to support this

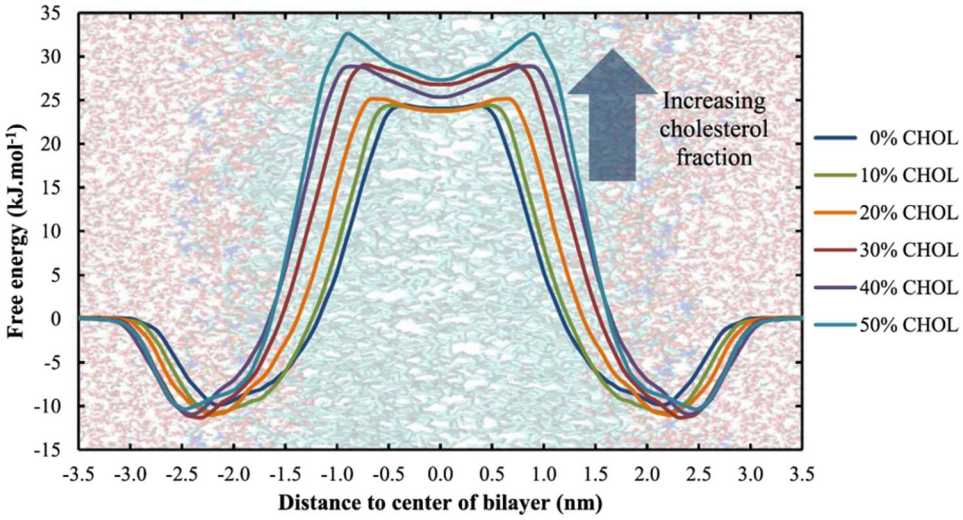


FIGURE 8 Effect of cholesterol fraction in cell membrane on the potential of mean force (PMF) of H_2O_2. With the permission of Van der Paal et al.[124]

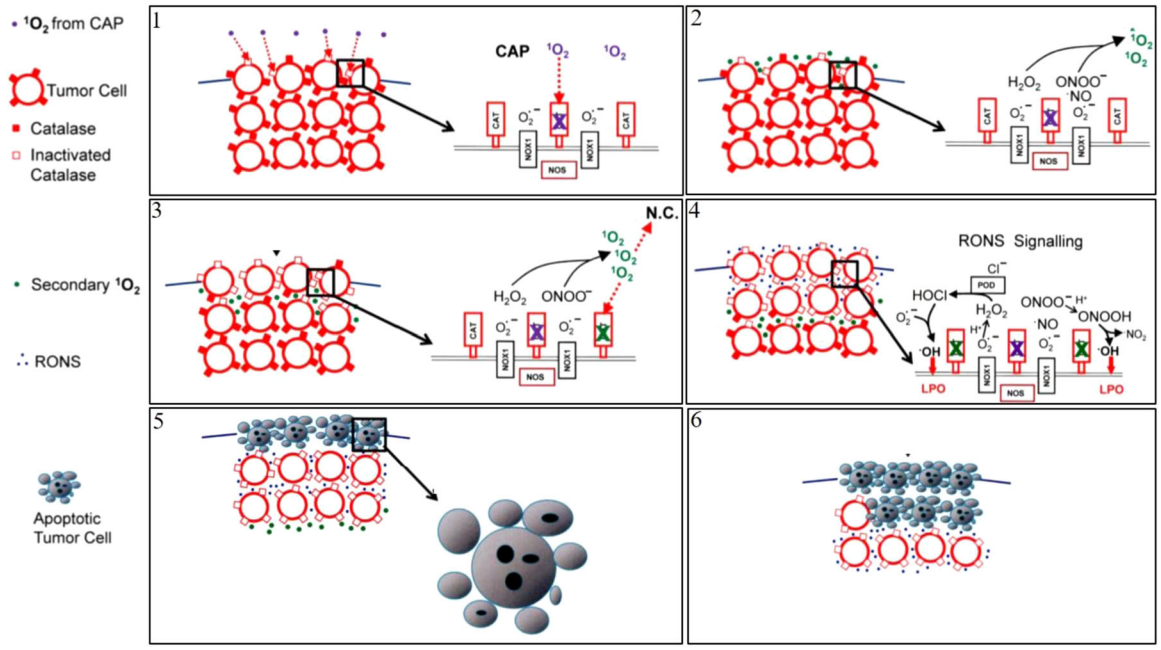


FIGURE 9 Hypothetical model to explain self-perpetuation of $^1O_2$ -triggered cell death. The model follows the order from (1)–(6). A detailed illustration is presented in the text. Permission from the Bauer and Graves[128]

explanation. Another caveat is the unclear role of $^1O_2$ in CAP cancer treatment. More experimental evidence is still needed to demonstrate that $^1O_2$ indeed plays a crucial role, at least as important as H_2O_2, to determine the final fate of CAP-treated cancer cells. In contrast, the critical role of H_2O_2 has been widely acknowledged in plasma medicine based on a large number of experimental evidence.[27,28,44,51,65,67,79–81] Furthermore, $^1O_2$ is a short-lived reactive species, with a half-life of several microseconds in water.[40] Thus, $^1O_2$ is not expected to play a role in the anticancer selectivity associated with CAP-activated solution or medium, which theoretically only allows long-lived reactive species and other reaction products to exert a biological effect on cancer cells (Table 2).[24,59,60,83,95–98] Thus, $^1O_2$ may play its unique role during direct CAP treatment in vitro or in vivo.

3.5 | Genomic repairing system

DNA damage is an important mechanism to trigger apoptosis in the CAP-treated cancer cells, which has been widely observed as an early event after treatment.[15,78] Double-strand break (DSB) is a main DNA damage style.[57,129] An important marker of DSB is the phosphorylation on serine 139 on H2AX histone (γ-H2AX), which has been widely observed immediately following CAP treatment.[57,129,130] The increased expression of phosphorylation of p53 (p-p53) in the CAP-treated melanoma cells (B16F10) was followed by the expression γ-H2AX, which follows the standard chronological order of DNA damage-triggered apoptosis pathways.[50] However, if DNA damage can be timely repaired, apoptosis may not be triggered. In mammalian cells, a fine genome repairing system can resist DNA damage to some extent.[131] In normal cells, the inefficient DNA damage repairing may accumulate mutations to initiate tumorigenesis or apoptosis.[132,133] Like normal cells, serious DNA damage in cancer cells will also trigger apoptosis. However, insufficient DNA damage repair mechanism in cancer cells facilitates tumor growth and aggression by accumulating mutations.[134–136] In many tumors, the genome repairing system has been compromised, facilitating the increased frequency of selective DNA damage and apoptosis in the CAP-treated cancer cells.[137]

4 | BEYOND CONVENTIONAL SELECTIVITY

4.1 | Coculture of cancer and normal cells

The traditional selectivity concept is based on a single cell line's response to the CAP-originated reactive species. Such a definition of selectivity can go beyond several ways. Considering the clinical cases, the interface between cancer tissues and normal tissues may be just the place where cancer cells' selective death is needed. The

coculture system of cancer cells and normal cells may simulate such an interfacial environment. If cancer cells can consume ROS/RNS faster than normal cells, cancer cells may protect normal cells in a coculture system. For example, glioblastoma cell line U87MG consumes H_2O_2 much faster than normal astrocyte cell line hTERT/E6/E7.[15] While this hypothesis remains to be fully tested experimentally, it can be used to explain the selective anticancer performance of a coculture system composed of normal cell line L02 and liver cancer cells HepG2.[97] An optimum treatment dose could selectively trigger the significant apoptosis in cancer cells in that study, leaving normal cells with minor damage.[97]

Furthermore, the selectivity can also be achieved by the self-adaptive guiding of bulk CAP jet to the side of normal cells in a coculture system by the physical feedback mechanism of cells to plasma. In a recent new coculture system, glioblastoma cell line U87MG could direct a helium CAP jet either toward or away from normal astrocyte cell line hTERT/E6/E7 (Figure 10).[138] The presence of a grounded copper board beneath the cell culture dish could overturn CAP jet's behaviors compared with a floating condition without such a copper board.[138] The cells' capacitance may be an essential factor to affect CAP jet's behavior.[138] This is the first demonstration that CAP jet's direction can be manipulated based on the permittivity difference between cancer cells and normal cells. This understanding may build the foundation to guide CAP selectively toward cancer tissue at the vicinity of normal tissues and realize a novel concept of selectivity based on the physical interaction between bulk CAP and cells.

4.2 | Specific cell-based H_2O_2 generation

Recent observations might provide yet another opportunity for the concept of selectivity. CAP sources have been regarded as the sole source of ROS, particularly H_2O_2, to affect cells' function and final fate. The discovery of cell-based H_2O_2 generation at the micromolar level challenges this consensus. H_2O_2 is an important signaling molecule in cancer cells.[37] About two decades ago, it was found that ovarian carcinoma, neuroblastoma, melanomas, and colon carcinoma can generate nanomolar levels of H_2O_2.[139] So far, CAP may be the sole tool to trigger cancer cells to generate a micromolar level of H_2O_2.[140] This discovery is unique in terms of plasma medicine and provides an interesting novel observation for cancer biology.

The cell-based H_2O_2 generation was initially observed in a triple-negative breast cancer cell line MDA-MB-231 and a pancreatic adenocarcinoma cell line PA-TU-8988T cells by comparing the H_2O_2 concentration generated in

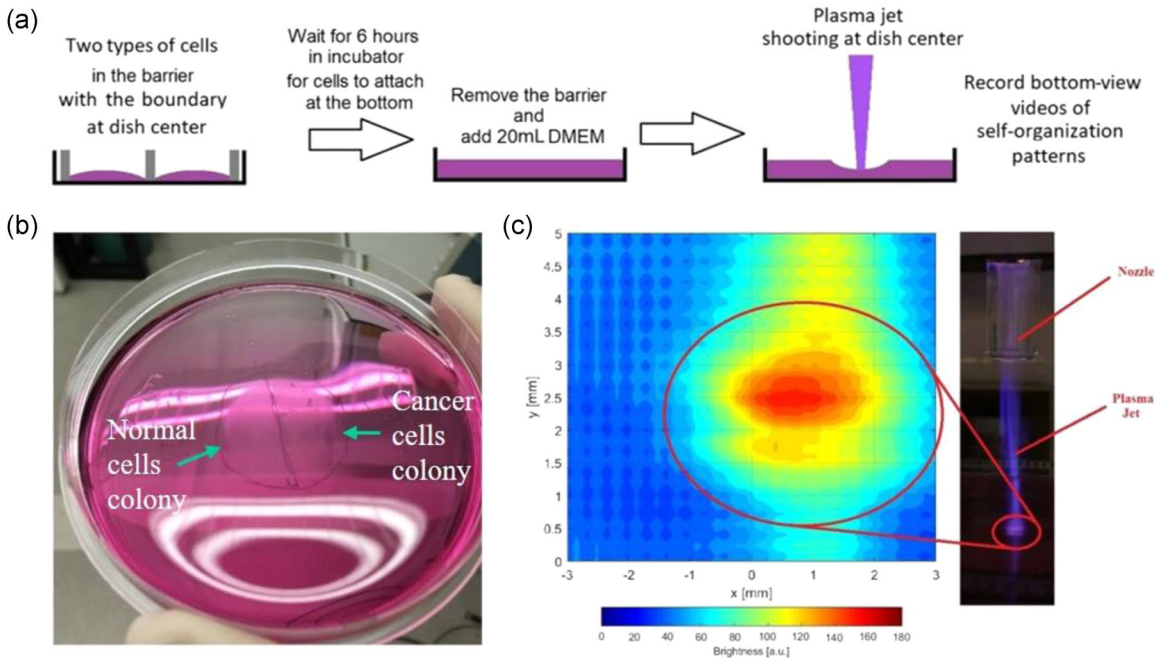


FIGURE 10 Bulk cold atmospheric plasma (CAP) jet was directed to the boundary of normal cell colonies and cancer cell colonies in a coculture system. (a) Schematic illustration of coculture system and experimental designs. (b) Photo of cell colonies in 100 mm culture dish. (c) A side-view image of the contact area of the plasma jet on targets. Normal astrocytes hTERT/E6/E7 and glioblastoma cells U87MG have grown on the negative-x (left) region and the positive-x (right) region, respectively. Permission from Lin et al.[138]

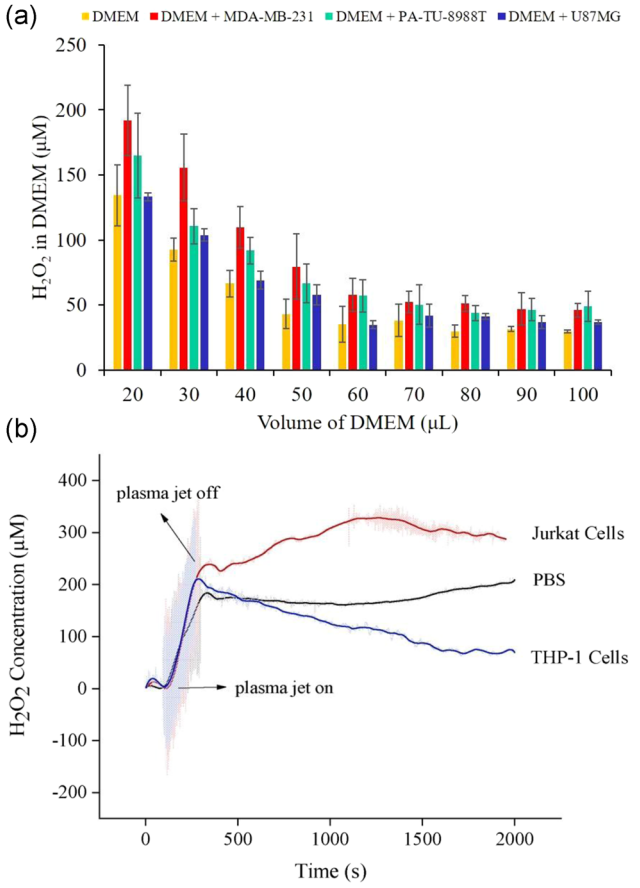


FIGURE 11 Specific cell-based H_2O_2 (micromolar) generation. (a) Direct cold atmospheric plasma (CAP) treatment can specifically trigger strong H_2O_2 generation in an extracellular environment. H_2O_2 concentration in Dulbecco's modified Eagle's medium (DMEM) was measured immediately (<1 min) after treatment. Each test with a specific volume of DMEM was performed based on four experimental designs: just DMEM, no cells (control), pancreatic adenocarcinoma cells (PA-TU-8988T) in DMEM, breast adenocarcinoma cells (MDA-MB-231) in DMEM, as well as on glioblastoma cells (U87MG) in DMEM. Permission from Yan et al.[140] (b) In situ H_2O_2 measurement over 3 min Ar plasma jet treatment and 30 min after the treatment. Jurkat T cells and THP-1 cells were human monocytic cell lines, and the cancer cell lines were derived from an acute monocytic leukemia patient, respectively. PBS represents the same measurement performed in control (pH 7.4). Permission from Nasri et al.[142]

the CAP-treated medium without and with cells.[140] When the volume of medium was adequately small, cell-based H_2O_2 generation could reach about 60 μM (Figure 11a). The discharge voltage is a vital parameter to modulate cell-based H_2O_2 generation. When the discharge voltage is adequately large, noticeable cell-based H_2O_2 generation can be observed in six other cancer cell lines, including breast cancer cell line MCF7, cervical adenocarcinoma cell line HeLa, bone osteosarcoma cell line U-2 OS, lung carcinoma cell line A549, colorectal carcinoma cell line HCT116, and melanoma B16F10.[141] In other cell lines, similar phenomena have been observed by another team using different methods, such as in situ H_2O_2 measurement after CAP treatment (Figure 11b).[142]

The cell-based H_2O_2 generation may be cell line-specific and selective. It is found that different cancer cell lines have quite different cell-based H_2O_2 generation under the same experimental conditions.[142] Compared with cancer cell lines, two normal cell lines, astrocyte cell line (hTERT/E6/E7) and fibroblast cell line (WDTF), generate only a low concentration of H_2O_2 (<5 μM) under same discharge conditions.[143] Similarly, the in situ measurements also showed that both Jurkat T cells, an immortalized human CD4+ T lymphocyte cell line (DSMZ) and THP-1 cells, a human monocytic cell line derived from an acute monocytic leukemia patient (CLS) could generate H_2O_2 compared with control. However, Jurkat T cells continue to generate H_2O_2 after CAP treatment. In contrast, THP-1 cells will quickly consume the extracellular H_2O_2 after CAP treatment (Figure 11b). A similar quick consumption has also been observed in our observation on PA-TU-8988T cells and MBA-MD-231 cells.[140] Such a selective cell-based H_2O_2 generation may also contribute to selectivity based on direct CAP treatment.

4.3 | The selectivity based on physical factors

The biological effect of CAP's physical factors, such as UV, thermal effect, and electromagnetic (EM) irradiation has been rarely observed in plasma medicine, particularly in cancer treatment for years. Thus, these physical factors have been regarded as a neglected role in the CAP's biological effect. Recently, the observation of cell death and sensitization by EM emission from CAP provided a new vision to understand the whole interaction between CAP and cells. When all chemical factors in CAP have been blocked in a novel experimental design, a strong physical effect could be observed. The EM emission from the CAP jet could penetrate the bottom of standard cell culture dish or multiwell plates, which can cause strong physically triggered cell death. The bottom was made of compact polystyrene material and had a thickness of around 1 mm. This physical barrier blocks all chemical factors, particularly reactive species, from contacting the cells on the other side. The cell death triggered by physical factors is characterized by cytosol aggregation and apparent bubbling on the cytoplasmic membrane, which is a process just lasting about 10 min.[144,145] Because the physically based CAP

treatment is essentially different from the chemically based treatment, one might expect that different cellular responses are involved. For glioblastoma U87MG cells and normal astrocyte cell line hTERT/E6/E7, the normal cell line is very sensitive to the reactive species' cytotoxicity. Thus, only a negative selectivity could be observed, which means normal cell lines will die more than cancer cell lines.[146] This limitation can be overcome as long as the dominant factors were reactive species. In contrast, the physically based CAP treatment can reinstate a positive selectivity toward glioblastoma cells by physical mechanism and physically triggered cell death (Figure 12).[146] To date, only this single study has been reported. It is promising to find more evidence that the physically based CAP treatment may also contribute to selectivity by an unprecedented mechanism.

For CAP-based cancer treatment discussed so far, nearly all studies focused on killing cancer cells directly by chemical or physical factors in CAP. This is also the basic motivation and strategy to perform plasma medicine-related studies in most cases. Recently, a novel work sheds light on a fully different routine to use CAP in cancer treatment. Not aiming to kill cancer cells directly, the discharge tube focuses on sensitizing cancer cells to the cytotoxicity of drugs in a contactless and transbarrier way, which could provide a promising new approach to affect deep subsurface tumor tissues, such as brain tumors. In a preliminary study, the helium gas was discharged in a sealed glass tube under atmospheric pressure conditions, referred to as the discharge tube. Compared with the CAP jet source, the discharge tube was essentially the same except that helium was sealed in a tube rather than being continuously supplied from the pressurized helium tanks (Figure 13a,b).[147] Two glioblastoma cell lines U87MG and A172 could be effectively sensitized to the cytotoxicity of a widely used glioblastoma drug temozolomide (TMZ) (Figure 13c). Interestingly, there was no apparent effect on normal astrocyte cell line hTERT/E6/E7 (Figure 13d). EM emission from the discharge tube triggered the sensitization, though its physical basis and underlying mechanism were still unknown.[147] The sensitization could be achieved when there was a macroscale air gap (5 mm) and the physical barrier of multiwell plates for cell

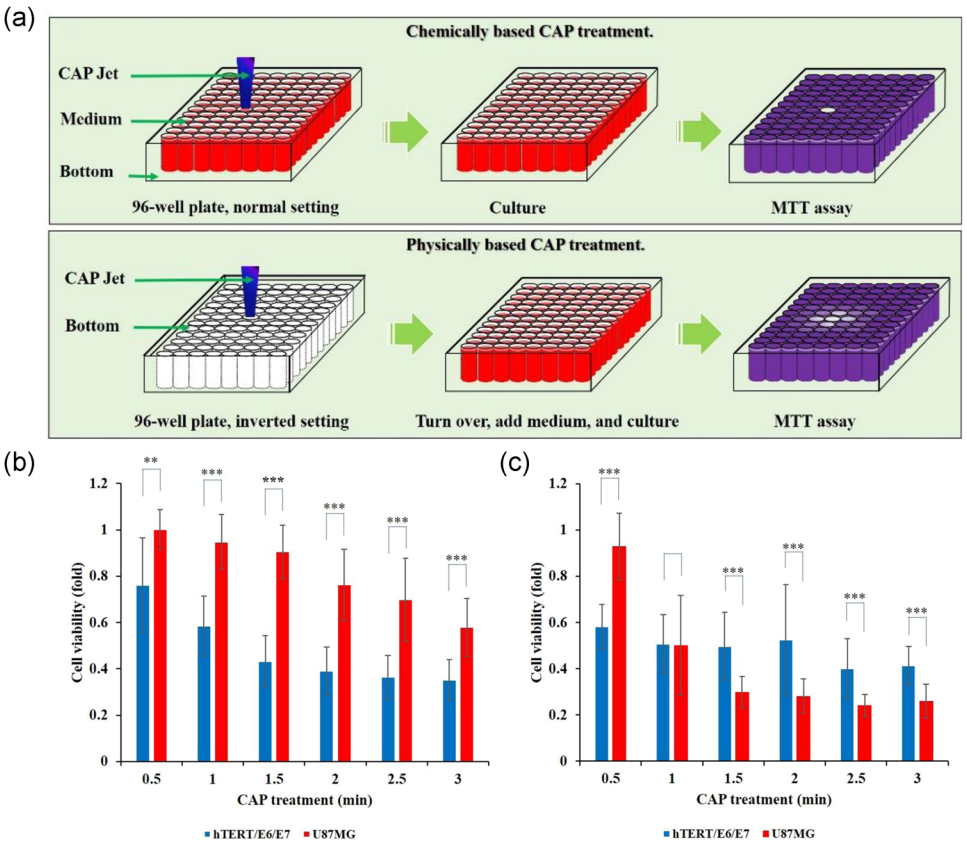


FIGURE 12 Physical treatment can reinstate the selectivity in cold atmospheric plasma (CAP)-treated astrocytes. (a) Schematic illustration of chemically based and physically based treatment. (b) Negative selectivity in chemically based treatment. (c) Positive selectivity in physically based treatment. Student's t-test was performed, and the significance was indicated as $*p < .05$, $**p < .01$, $***p < .005$. Permission from Yan et al.[146]

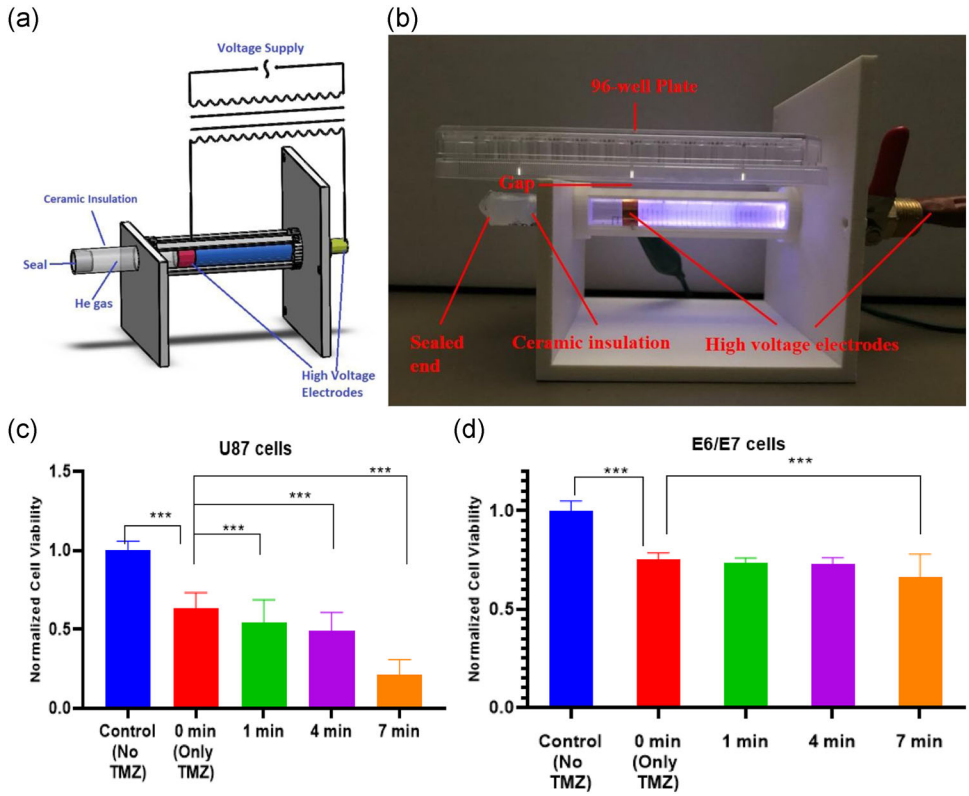


FIGURE 13 Selective sensitization on glioblastoma cells by a helium gas discharge tube. (a) The schematic illustration of setup. (b) The photo of the discharge tube in operation. (c) Strong sensitization of glioblastoma U87MG cells to temozolomide (TMZ) (250 μM). (d) Weak impact on normal astrocyte cell line hTERT/E6/E7. (Student's t-test, ***$p < .05$). Permission from Yao et al.[147]

culture. Sensitization is also referred to as activation and can be achieved by a typical CAP jet source. In fact, the activation phenomena were first discovered during the direct CAP treatment using a helium CAP jet. In that case, pancreatic cancer cells were sensitized to the cytotoxicity of long-lived reactive species, such as H_2O_2 and NO_2^-.[148,149] The activation mechanism is also unknown. However, the short-lived reactive species and physical factors rather than long-lived reactive species may cause the activation in direct treatment.

5 | CONCLUSIONS

In this review, the traditional concept of CAP-triggered selective death of cancer cells has been regarded as a narrow concept of selectivity. It focuses on the single-cell line's death rate to single CAP treatment on this cell line, which is fully determined by CAP's reactive species. AQP, antioxidant system, transition pore formation, and cellular membrane component, as well as singlet oxygen, may contribute to such a selective death of cancer cells upon CAP treatment. It is necessary to note that selectivity will not definitely occur in all cases. This is most simply explained by differences in the expression patterns of individual proteins or protein families in cancer cells and their normal counterpart cells. Take the AQP-based explanation as an example, if specific cancer cells express AQP 1, 3, 8, or 9 less than normal cells, CAP may kill cancer cells with negative selectivity. Similarly, for the explanation based on transition pore formation on the cellular membrane, if the specific cancer cells have more cholesterol in the cellular membrane than normal cells, negative selectivity may also occur. In short, the selective anticancer capacity should not be regarded as a single factor determined.

Beyond the traditional definition of selectivity, we provided some new visions for selectivity's general concept. A coculture system, for example, involves a cancer cell line and a normal cell line simultaneously. Even though the selectivity does not occur on a single cell line, the coculture of a cancer cell line with another normal cell line may protect normal cells by the higher reactive species' consumption speed. The discovery of the capacitance-based physical feedback mechanism of cells to bulk CAP jet for the first time provides an exciting routine to guide CAP to treat cancer cells in the presence of neighboring normal cells. The new studies of physical effects on cancer cells, including the direct killing effect and the sensitization effect, provide unprecedented

selectivity concepts. Physically based CAP treatment can generate selectivity on glioblastoma cells, which cannot be achieved by traditional chemically based CAP treatment. The discharge tube, a novel CAP source, can sensitize glioblastoma cells to the cytotoxicity of the widely used drug TMZ without causing side effects on normal astrocytes. These new results provide a clear vision that CAP treatment's selectivity can extend its application beyond a superficial understanding, which was limited mainly to a pharmaceutical-like approach. So far, the clinical application of CAP in cancer treatment is mostly unclear. Finding possible new routines to use CAP in cancer treatment will facilitate the ultimate realization of clinical CAP cancer treatment.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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