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yan Zang
First Affiliated Hospital of Anhui Medical University
Zhimin Ding
The People's Hospital of Xuancheng City
Chengbiao Ding
The Second Hospital of Anhui Medical University
zongshi Gao
First Affiliated Hospital of Anhui Medical University
dongyu SI
First Affiliated Hospital of Anhui Medical University
zhan Gao
First Affiliated Hospital of Anhui Medical University
zhengwei Wu
University of Science and Technology of China
huabing Zhang
Anhui Medical University
chaobing Gao
gaochaobing@ahmu.edu.cn

First Affiliated Hospital of Anhui Medical University

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Reactive Oxygen Species–Driven Mitochondrial Apoptotic Signaling and ER Stress Responses in HNSCC Cells Treated with Cold Atmospheric Plasma

Yan Zang[1,2],Zhimin Ding[5],Chengbiao Ding[6],Zongshi Gao[1],Dongyu Si[1],Zhan Gao[1,2], Zhengwei Wu*[4],Huabing Zhang*[3], Chaobing Gao*[1]

1. Department of Otorhinolaryngology Head and Neck Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, 230022, China.
2. Department of Otorhinolaryngology Head and Neck Surgery, Anhui Public Health Clinical Center, Hefei 230012, China.
3. Department of Biochemistry & Molecular Biology, Metabolic Disease Research Center, School of Basic Medicine, Anhui Medical University, Hefei, 230022, China.
4. School of Nuclear Science and Technology, University of Science and Technology of China, Hefei, 230022,China
5. Department of Otorhinolaryngology Head and Neck Surgery, The People's Hospital of Xuancheng City, Xuancheng, 242000, China.
6. Department of Rehabilitation Medicine, The Second Hospital of Anhui Medical University, Hefei,230022, China

Corresponding authors:

Zhengwei Wu,Ph.D,School of Nuclear Science and Technology, University of Science and Technology of China,Hefei,230022,China.E-mail: wuzw@ustc.edu.cn.

Huabing Zhang,Ph.D,Department of Biochemistry & Molecular Biology, Metabolic Disease Research Center,School of Basic Medicine,Anhui Medical University, Hefei,230022,China. E-mail:huabingzhang@ahmu.edu.cn;

Chaobing Gao,MD.Department of Otorhinolaryngology Head and Neck Surgery,The First Affiliated Hospital of Anhui Medical University,Hefei,230022,China. E-mail: gaochaobing@ahmu.edu.cn.

Abstract:

Objective: Head and neck squamous cell carcinoma (HNSCC) remains a challenging cancer with poor survival and significant treatment-related morbidity. Cold atmospheric plasma (CAP) has potential anticancer effects; however,its specific molecular mechanisms in HNSCC remain incompletely understood. This study examined the antitumor actions of CAP on HNSCC together with associated ROS-mediated mitochondrial pathways.

Methods:Two HNSCC cell lines (FaDu and TU212) were treated with CAP for varying durations (0–240 s). Cell viability and migration were evaluated using CCK-8 and wound-healing assays, respectively, while apoptosis was examined using flow cytometry. Intracellular ROS levels were quantified. The activation of mitochondrial apoptotic pathway was evaluated by Western blotting for key markers (Bax,Blc-2 and cleaved caspase-3) and transmission electron microscopy. Furthermore, antitumor effects were verified using a FaDu xenograft mouse model, with tumor growth,proliferation (Ki-67),and apoptosis(C-Caspase-3) analyzed via immunofluorescence.

Results: CAP administration led to marked time-dependent reductions in HNSCC cell viability and migration.Mechanistically, CAP persistently elevates intracellular ROS, triggering mitochondrial pathway-mediated apoptosis and ER stress, and marked ultrastructural changes including ER vacuolization and nuclear fragmentation. In mice, CAP administration markedly suppressed tumor growth without causing systemic toxicity or skin damage, correlating with reduced proliferation and enhanced apoptosis within the tumor microenvironment.

Conclusion: CAP exerts potent antitumor effects against HNSCC by inducing a self-amplifying oxidative stress response that activates endogenous apoptosis and endoplasmic reticulum stress. These results indicate the potential of CAP in treating HNSCC and provide a mechanistic rationale for its future clinical translation.

Keywords: Cold atmospheric plasma (CAP); Head and neck squamous cell carcinoma (HNSCC); Endoplasmic reticulum stress; Reactive oxygen species (ROS); Apoptosis;

1. Introduction

Head and neck cancers impose a formidable global health challenge, with annual statistics indicating approximately 700,000 new diagnoses and 380,000 fatalities, a trend that continues to rise [1]. Head and neck squamous cell carcinoma (HNSCC) represents an estimated 90% of these tumors. HNSCC typically originates from laryngeal, pharyngeal, and oral squamous epithelia， while some tumors involve the nasal cavity, paranasal sinuses, and salivary glands [2].

Therapeutic management of HNSCC is complicated by the region's intricate anatomy, where respiratory and digestive pathways converge,and by the critical need to preserve aesthetic appearance and physiological function. Although multimodal strategies integrating surgery, radiotherapy, chemotherapy, and targeted therapies have advanced,the 5-year survival rate has stagnated over the last decade [3]. Advanced-stage patients face grim prognoses characterized by high rates of local recurrence and metastasis. Moreover, survivors often suffer from severe sequelae, including dysphagia and dysarthria, which drastically reduce quality of life. The emergence of intrinsic or acquired chemoresistance further exacerbates the clinical burden, underscoring the urgent necessity for novel therapeutic modalities.

Plasma represents a state of matter distinct from solids, liquids, and gases, and arises from ionization of neutral gases under strong electric fields. This process yields a complex milieu of ions, electrons, neutral species, reactive free radicals, and ultraviolet radiation [4].Unlike thermal plasma, Cold Atmospheric Plasma (CAP) operates under non-equilibrium conditions, maintaining heavy particles (ions and neutrals) at near-ambient temperatures while generating highly reactive chemical species [5]. This thermal compatibility with biological tissues has propelled CAP into the biomedical arena [6,7].

Clinical adoption of CAP devices has already proven successful in treating infected wounds and dermatological infections, yielding favorable outcomes [8,9].The scope of CAP applications is rapidly expanding; initial experimental investigations in 2007

revealed potent tumoricidal effects against malignant melanoma [10].Subsequent studies have corroborated CAP's anticancer efficacy across a spectrum of malignancies, including glioma, lung, prostate, cervical, and breast cancers [11]. CAP has shown significant potential as a cancer treatment. Previous studies have shown that the primary mediators of these effects are reactive oxygen and nitrogen species (RONS). reactive oxygen species(ROS), as an important and ubiquitous second messengers in living organisms, stimulate intracellular signaling at low concentrations but exert cytotoxic effects at high concentrations[12]. However, the key biological effectors appear to be not the primary species generated by plasma, but rather the secondary products and ROS derived from these primary species after interacting with the organism [13].Consequently, elucidating the molecular mechanisms governing CAP-induced tumor suppression remains a pivotal research focus.

In this study, we used both cellular and animal models to evaluate the antitumor efficacy of CAP against HNSCC and to dissect the underlying biological mechanisms.

2. Materials and Methods

2.1 CAP Device Configuration

FaDu and TU212 cells were analyzed using a custom-fabricated plasma device capable of high-throughput 96-well plate processing（Figure1. A-E）.The device measures approximately 15 mm× 10 mm. Two electrodes were constructed via micro-arc oxidation and plasma coating, as detailed in Ding's prior work〔14〕. Operating in a pulsed discharge mode with ambient air as the working gas, the device maintained a surface temperature below 30°C, preventing thermal damage and reducing energy consumption.

For in vivo applications, a distinct configuration was adopted to address plasma penetration limitations (Figure1.F). A needle electrode, insulated except at the tip, was inserted subcutaneously above the tumor in murine models. This electrode was connected to the plasma generator via a copper sheet, with 4 mL of ambient air injected as the working gas. A grounding electrode was attached to the left hind limb

of each mouse. Despite structural differences between the cellular and animal models, key operational parameters, including discharge mode, operating temperature, and excited state generation, were consistent. The discharge duration was standardized at 120 seconds.

2.2 Cell Culture and CAP Treatment Protocols

FaDu cells (iCell Bioscience Inc.) were maintained in DMEM, while TU212 cells (BeNa Culture Collection) were cultured in IMDM, both with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2.

Prior to CAP exposure, culture media were aspirated. Cells were stratified into four groups based on treatment duration: 0s (control), 60s, 120s,and 240s. The plasma source was positioned 10 mm above the monolayer, and standard voltage was applied. Following irradiation, fresh medium was added to the cells, which were then grown for 24 h.

In animal studies, anesthetized mice were secured on a platform every other day. The needle electrode was inserted subcutaneously, air was introduced, and plasma was generated via arc discharge surrounding the tumor mass.

2.3 Xenograft Tumor Model Establishment

BALB/c nude mice (male, five weeks old) were maintained in SPF facilities at 24°C and were rendomized into control and experimental groups (n = 5 per group). Exponentially growing FaDu cells (4×10^5 in 100 μL PBS) were inoculated subcutaneously into the right flank. Upon reaching a tumor volume of ~100 mm^3, treatments commenced. Tumor dimensions and body weights were determined on alternate days.The experimental cohort received CAP treatment five times over an 18-day period. All animals were sacrificed on day 19. Pentobarbital sodium was used as the anesthetic agent (50 mg/kg, intraperitoneal injection) before CAP treatment, and anesthetic depth was confirmed by the loss of the pedal withdrawal reflex. Prior to tumor harvesting, mice were first anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal injection); after deep anesthesia was confirmed by the loss of the pedal withdrawal reflex, mice were euthanized by rapid cervical dislocation performed by trained personnel.The protocol was approved by the Animal Ethics Committee of Anhui Medical University (No.20232047) and mice were sourced from GemPharmatech Co. Ltd.

2.4 Cell Viability and Ultrastructural Analysis

Cytotoxicity was quantified using CCK-8 assays. Cells (5×10^3/well) were inoculated in 96-well plates and grown overnight. Following medium removal and CAP irradiation, cells were cultured for 24 hours in fresh medium. CCK-8 solution (10 μL) was introduced 1 hour prior to measurement, and absorbance was recorded at 450 nm. Additionally, transmission electron microscopy (TEM) was utilized to examine ultrastructural changes.

2.5 Colony formation assay

Approximately 1,500 cells were seeded per well in a 6-well plate. After cell attachment to the culture vessel wall, the control group was treated with 0.1% Dimethyl Sulfoxide (DMSO), while the experimental groups were treated with CAP for 60s, 120s, and 240s, respectively, followed by replacement with fresh medium. On day 7, cells were fixed with 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet for 30 min, and then observed using a digital camera.

2.6 Wound Healing Assays

Cell migratory potential was examined via wound-healing assays. Following 120s of CAP treatment, cells were inoculated in 6-well plates in serum-free medium. A linear scratch was made using a 100 μL pipette tip. The cell monolayer was monitored at 0, 6, 12 and 24hours using an inverted microscope to assess gap closure.

2.7 ROS Detection

Intracellular ROS contents were examined using a DCFH-DA probe and flow cytometry. Post-CAP, cells were harvested, rinsed with PBS, and treated with diluted DCFH-DA (1:1000) at 37°C for 20 minutes with gentle shaking every 5 minutes. After three PBS rinses, fluorescence intensity was determined via flow cytometry. Comparisons were made between immediate post-treatment and 24-hour post-treatment time points.

2.8 Apoptosis

An Annexin V-FITC/PI Apoptosis Detection Kit (Wanleibio) was used to assess apoptosis. 24 hours post-CAP, cells were harvested, rinsed with PBS, and suspended in 500 μL binding buffer before treatment with 5 μL FITC and 10 μL PI for 15 minutes at room temperature while protected from light, and evaluated immediately on an Agilent NovoCyte flow cytometer.

2.9 Immunofluorescence Staining

Tumor samples were fixed (4% paraformaldehyde, 48 hours), paraffin-embedded, and sectioned (5μm).After antigen retrieval and blocking 5% goat serum, tissues were treated overnight with antibodies against cleaved-caspase-3 and Ki67 at 4°C. Following secondary antibody incubation (37°C, 1.5 hours) and DAPI counterstaining, images were acquired using an immunofluorescence microscope.

2.10 Statistical analyses

Dates are means ± SD and were analysed with GraphPad Prism 6.0. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001,ns: no significance.

3. Results

3.1 CAP Exerts Cytotoxicity on HNSCC Cells

Flow cytometry analysis revealed that cell viability was markedly and time-dependently reduced following CAP treatment（figure 2.A）. In FaDu cells, viability dropped from 63.1% ± 7.8% (control) to 34.1% ± 3.4% (CAP60s), 27.8% ± 3.6% (CAP120s), and 18.6% ± 3.0% (CAP240s). Similarly, TU212 cells exhibited a decline from 55.9% ± 6.4% (control) to 31.1% ± 2.7% (CAP60s), 25.8% ± 3.6% (CAP120s), and 18.1% ± 2.4% (CAP240s). These data confirm that CAP induces potent cytotoxicity in HNSCC cells in a time-dependent manner. Colony formation assay yielded consistent results, demonstrating that CAP inhibited the proliferative capacity of HNSCC cells in a time-dependent manner(figure2.B-C). Notably, cell viability continued to decline during the post-treatment incubation period following 60s of

CAP exposure(figure2.D). This sustained reduction is attributed to the generation of reactive oxygen and nitrogen species (ROS/RNS) induced by CAP treatment.

3.2 CAP Inhibits Migration in HNSCC Cells

Wound-healing assays indicated significant impairment of cell motility following CAP treatment（figure3.）. While control groups showed rapid scratch closure over 24 hours, CAP-treated groups (CAP120s) exhibited markedly delayed healing rates. This inhibitory effect persisted and widened over time, indicating that CAP effectively suppresses the migratory capacity of HNSCC cells even after the cessation of treatment.

3.3 CAP Triggers Apoptosis in HNSCC Cells

Flow cytometry analysis confirmed that CAP suppresses proliferation by inducing apoptosis（figure4.）. In FaDu cells, the apoptotic fraction increased from 4.27% (control) to 32.88% (CAP60s), 40.94% (CAP120s), and 60.74% (CAP240s), with a concomitant decrease in viable cells. Parallel trends were observed in TU212 cells, where apoptosis rose from 4.36% (control) to 29.76% (CAP60s), 42.23% (CAP120 s)and 57.21% (CAP240s). These results underscore the pro-apoptotic efficacy of CAP (Figure 4B).

3.4 CAP Elevates Intracellular ROS in HNSCC Cells

Flow cytometric analysis showed marked time-dependent increases in intracellular ROS concentrations in FaDu and TU212 following CAP treatment, evidenced by a rightward shift in FITC fluorescence intensity(figure 5.). Quantification of Median Fluorescence Intensity (MFI) confirmed that ROS accumulation was statistically significant compared to controls. Notably, ROS levels measured 24 hours post-treatment were higher than those detected immediately after exposure, suggesting that CAP triggers a sustained oxidative stress response that intensifies over time.

3.5 CAP Induces Morphology Alterations in HNSCC Cells(FaDu)

TEM analysis of FaDu cells revealed distinct morphological changes（figure 6.）. Control cells displayed intact plasma membranes and nuclear envelopes with normal mitochondrial distribution（figure6.A）.In contrast, the 60s-CAP group exhibited early apoptotic features, including chromatin condensation at the nuclear periphery and Disappearance of mitochondrial cristae, Endoplasmic reticulum rupture and local dilation（figure6.B）. After CAP treatment for 120 sec,FaDu shows More pronounced apoptotic signatures,such as chromatin condensation,Mitochondrial swelling, endoplasmic reticulum dilation extensively, widening of the perinuclear spaceetc(figure6.C). Prolonged exposure (240s) resulted in severe ultrastructural damage characteristic of late-stage apoptosis: nuclear shrinkage， extensive ER vesiculation(figure6.D).

3.6 CAP Suppresses Tumor Growth In Vivo

In the FaDu xenograft model, administration of CAP markedly reduced tumor growth relative to the control group, without causing significant body weight loss or visible skin damage. These findings demonstrate the potent antitumor activity and favorable safety profile of CAP in vivo(figure7.C-G).

3.7 CAP Modulats Proliferation and Apoptosis In Vivo

Immunofluorescence staining of tumor sections revealed a substantial reduction in Ki-67 expression (proliferation marker) and increases in cleaved caspase-3-positive cells (apoptosis marker) following CAP administration. These results corroborate the in vitro findings, confirming that CAP suppresses tumor progression by inhibiting proliferation and inducing apoptosis (figure7.H-I).

4. Discussion

Laryngeal cancer and hypopharyngeal cancer are among the most common types of HNSCC treated in our Department of Otorhinolaryngology – Head and Neck Surgery. Although there is a significant difference in their 5-year survival rates, the prognosis for individuals with advanced disease is unfavorable for both types. These patients often suffer from tumor-related huge ulceration and infection, massive hemorrhage,

laryngeal obstruction, and dysphagia. Since the initial observation of plasma-induced melanoma apoptosis in the 1980s, CAP has shown promise in treating cancer, showing potential for both solid tumors, such as breast, thyroid, and pancreatic cancers, and hematological malignancies like multiple myeloma [15]. Early research primarily documented its cytotoxicity, whereas contemporary studies have shifted towards deciphering the molecular underpinnings, particularly the functions of RONS. Previous investigations have linked CAP to mitochondrial ROS-mediated MAPK signaling in head and neck cancer [16], MMP-9 modulation in HeLa cells [17], and G1 arrest mediated by the ATM/p53 axis in oral squamous cell carcinoma [18]. Crucially, CAP often exhibits selective toxicity towards tumor cells over normal counterparts, which is a key feature of CAP as a potential anti-cancer candidated drug [19, 20,21].

Here, we systematically characterized the influence of CAP on FaDu and TU212 cells. Our data reveal a clear time-dependent correlation in cell cytotoxicity and CAP exposure, a similar trend was also observed in intracellular ROS levels. Importantly, ROS levels continued to rise during a 24-h post-treatment incubation period compared with measurements taken immediately after CAP exposure, and show a higher upward trend,suggesting a cumulative and sustained intracellular oxidative response. This temporal persistence implies that CAP-derived reactive species may retain biological activity beyond the initial exposure window, thereby prolonging therapeutic efficacy. It has been found that CAP generates both short-lived radicals, including hydroxyl radicals (•OH), superoxide anions ($O2^{-}$), and singlet oxygen (O2), and long-lived forms, including hydrogen peroxide (H2O2), nitrite ($NO2^{-}$), and nitrate ($NO3^{-}$), in both gas and liquid phases[22]. These RONS are regarded as key mediators of CAP-induced biological effects. Excessive accumulation of such species disrupts cellular redox homeostasis, triggering oxidative stress and activating pro-apoptotic signaling cascades.

Consistent with this mechanism, CAP-treated HNSCC cells exhibited markedly reduced viability relative to untreated controls, indicative of apoptosis induction.

Because cellular migration is highly dependent on mitochondrial oxidative phosphorylation and ATP production-and closely associated with tumor invasiveness and metastatic potential-we further examined cell motility. In vitro migration assays demonstrated that CAP exposure significantly and persistently inhibited the migration capability of FaDu and TU212 cells. Cell migration is a critical step in tumor distant metastasis. The significant inhibition of FaDu and TU212 cell migration by CAP suggests its potential clinical application in preventing the spread of head and neck cancers，which aligns with findings in breast cancer models of Wang M et al [23].

Excessive oxidative stress is known to induce apoptosis and ER stress.This mechanism underpins the efficacy of various anticancer therapies, including chemotherapeutic agents (e.g. paclitaxel, bortezomib) [24,25] and photodynamic therapy (PDT) [26].As a pivotal organelle, the endoplasmic reticulum (ER) orchestrates critical cellular functions, such as protein synthesis and folding, post-translational modifications, preservation of calcium homeostasis, and lipid metabolism. Under pathological conditions—such as hypoxia, irradiation, or elevated ROS levels,ER stress can arise due to the presence of accumulated misfolded or unfolded proteins, inducing the UPR. Although the UPR is initially adaptive in nature, leading to restoration of cellular homeostasis, long-term or excessive ER stress induces apoptosis [27] . Importantly, cancer cells, which typically maintain higher basal ROS concentrations compared to normal cells, are particularly vulnerable to ER stress–mediated apoptosis [28] .

With the prolongation of cold atmospheric plasma (CAP) treatment time, FaDu cells exhibited an increasingly pronounced apoptotic trend under transmission electron microscopy (TEM). In untreated control cells, chromatin was evenly distributed, mitochondrial cristae were clear and intact, and the cell surface was rich in orderly-arranged microvilli. After 60s of CAP treatment, some mitochondrial cristae began to blur or disappear, and early marginal aggregation of chromatin appeared. When the treatment time was extended to 120s, electron microscopy revealed a marked intensification of chromatin condensation, accompanied by

mitochondrial swelling, endoplasmic reticulum (ER) dilation, ribosome detachment, and widening of the perinuclear space—all typical features of early- to mid-stage apoptosis. After a longer duration (240s) of CAP treatment, the nuclei underwent severe shrinkage,Endoplasmic reticulum (ER) vacuolization observed by transmission electron microscopy (TEM) is indicative of robust ER stress, and the mitochondria were extremely swollen with complete disappearance of cristae, exhibiting an irreversible apoptotic morphology. Western blot results further confirmed this process at the protein level: with the prolongation of CAP treatment time, the expression of Cleaved-Caspase 3 in FaDu and TU212 cells was significantly upregulated, clearly indicating activation of the execution phase of apoptosis. As the organelle most sensitive to ROS levels, mitochondria exhibited swelling and loss of cristae under TEM, strongly suggesting that mitochondrial dysfunction may be a key link in CAP-induced apoptosis. The classical mitochondrial apoptotic pathway involves the dynamic balance regulation of Bcl-2 family members. In this study, the expression levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were examined. The results showed that CAP treatment led to a significant downregulation of Bcl-2 expression and a marked upregulation of Bax expression, resulting in a substantial decrease in the Bcl-2/Bax ratio. This change indicates that Bcl-2 was unable to effectively inhibit Bax oligomerization and the increase in mitochondrial outer membrane permeability, thereby initiating the downstream cascade reaction that leads to apoptosis.

This conclusion is highly consistent with the findings of multiple previous studies across species and disease models. Strížová et al. found that CAP could induce apoptosis in yeast by triggering the mitochondrial pathway [29] . In the field of cancer research, Wang et al. demonstrated that CAP significantly reduces the mitochondrial membrane potential of osteosarcoma cells, promotes the release of cytochrome c from mitochondria into the cytoplasm, and thereby activates the caspase cascade, establishing the central role of the mitochondrial pathway in the anti-osteosarcoma effect of CAP [30]. In addition, the study by Ding et al. also confirmed that CAP can

similarly ameliorate the pathological progression of a rheumatoid arthritis model by regulating mitochondrial function, effectively alleviating the positive signs in rats, further corroborating the universality of mitochondria as a key target of CAP[31]. In summary, this study, together with previous work, collectively confirms that CAP induces programmed death in head and neck squamous cell carcinoma by disrupting mitochondrial homeostasis, regulating the Bcl-2/Bax balance, and activating caspase-3.

Verification in a xenograft model confirmed the antitumor effect of CAP observed in cellular models. CAP administration once every other day resulted in an visible reduction in tumor volume and weight compared with control animals without observable adverse effects such as skin damage or body weight loss. Immunofluorescence analysis of tumor tissues revealed decreased Ki-67 expression and increased cleaved-caspase-3 positivity in CAP-treated tumors, confirming suppression of proliferative activity and enhancement of apoptosis in vivo. These findings provide compelling evidence that CAP exerts antitumor activity with favorable tolerability.

Collectively, our study demonstrates that CAP suppresses HNSCC cell viability and migratory capacity through the induction of mitochondrial apoptotic pathways and endoplasmic reticulum stress(figure 8.). The observed time-dependent accumulation of intracellular ROS highlights CAP's ability to induce sustained oxidative stress, thereby amplifying apoptotic signaling. Importantly, these findings are consistent with and extend previous observations in other tumor models, including colon, thyroid, and breast cancers, suggesting that CAP launches conserved cellular stress-response mechanisms across malignancies [35, 36, 37]

Although the antitumor efficacy of CAP was validated in a FaDu xenograft mouse model, the sample size was relatively small, and only a single cell line was used for *in vivo* experiments. Larger cohorts and additional HNSCC cell lines are warranted to confirm the generalizability of our findings.While our data support the involvement of the ROS-mediated ER stress-apoptosis axis, the precise molecular interplay between

CAP-generated RONS and specific ER stress sensors remains to be fully elucidated. Further studies using pharmacological inhibitors or genetic knockdown are needed to dissect these pathways in greater detail. Finally, standardization of CAP device parameters and delivery protocols is essential for clinical translation, Future clinical trials are required to evaluate the safety and efficacy of CAP in human subjects.

5.Artical summary

In summary, the study findings indicate the potential of CAP in treating HNSCC. By inducing ROS-dependent mitochondrial dysfunction and activating the ER stress, CAP effectively triggers apoptosis, thereby inhibiting tumor growth and migration. These mechanistic insights, supported by robust in vivo evidence, highlight the translational promise of CAP and lay the groundwork for its integration into multimodal cancer treatment regimens.

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Author Contributions

Z.W.W., H.B.Z.and C.B.G. conceived and designed the study. Y.Z. and C.B.D. performed the cell-related experiments. Z.M.D. and Y.Z.performed the animal experiments. Z.S.G.performed the analysis of data. All authors checked the results and wrote the manuscript. All authors have read and approved the final manuscript.

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Ethics statement

No human subjects were involved in the study. All animal protocols followed the guidelines of the Animal Center of Anhui Medical University and were approved by the Animal Ethical Committee of Anhui Medical University.

Conflict of Interest

The authors declare no conflict of interest.

Data sharing statement

The data supporting the current study are available from the corresponding author upon reasonable request.

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Figures and captions

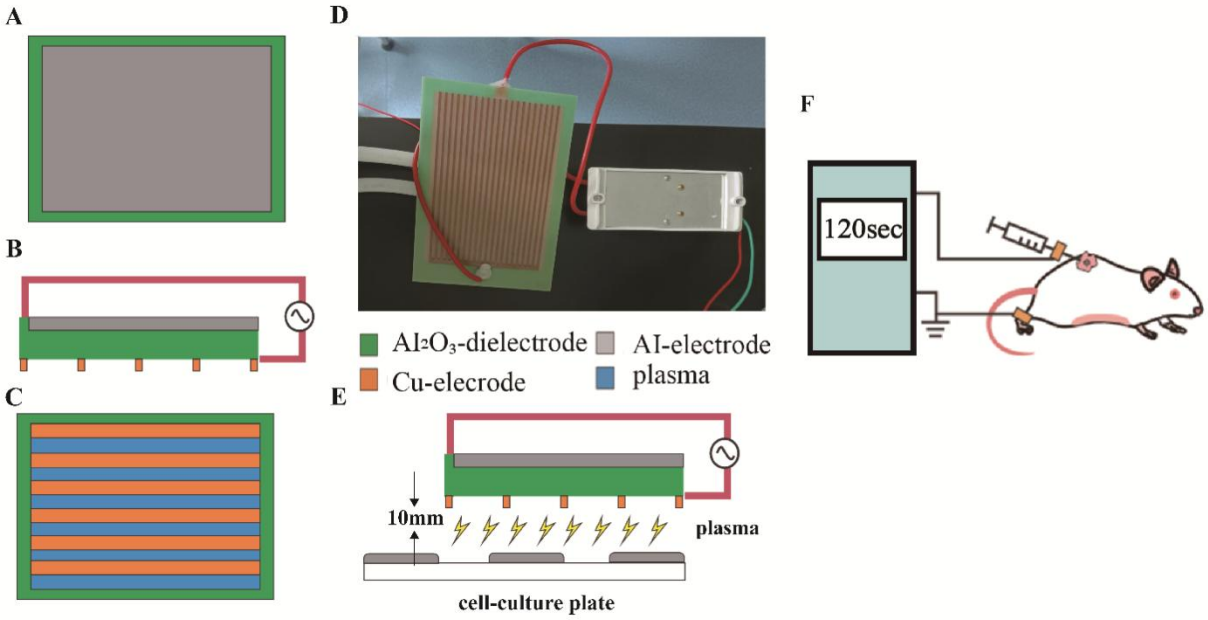


Fig1. Illustration of the cold atmospheric plasma (CAP) devices for tumor treatment in vitro(A-E) and in vivo(F).

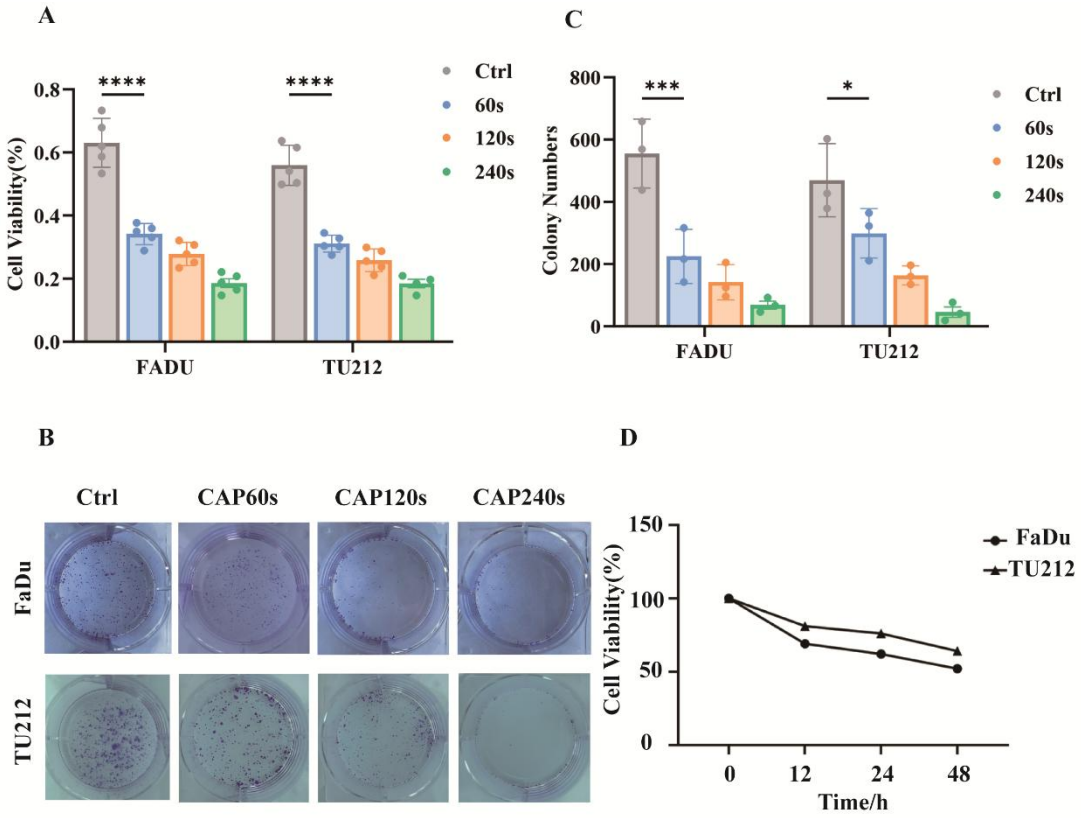


Fig2.CAP decreased the viability of HNSCC cells

（A）Cell viability assays showed that CAP treatment (60s,120s,240s) suppressed the viability of FaDu and TU212 cells; (B)Representative colony formation assays revealed that CAP suppressed the clonogenic potential of TU212 and FaDu cells, with the inhibition intensifying as the treatment duration extended.(C)Quantitative analysis of the inhibitory effect of CAP on colony formation in TU212 and FaDu cells across different treatment durations.(D) Following a 60s treatment with CAP, the viability of FaDu and TU212 cells decreased further as the incubation time was extended. compared with the control group, *P < 0.05, ***P < 0.001, ****P < 0.0001;All experiments were performed in at least three independent replicates.

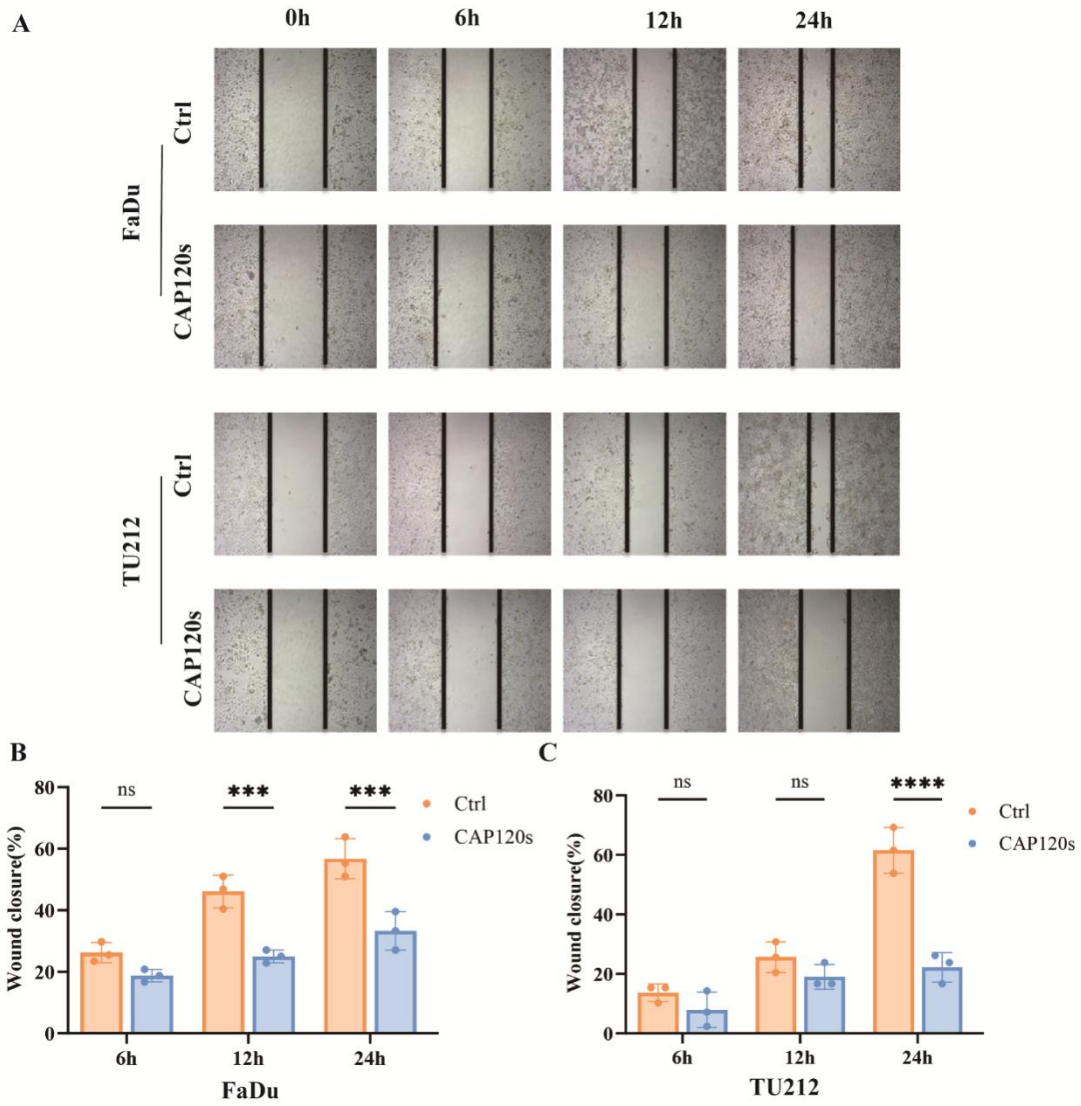


Fig3.CAP inhibited the migration of HNSCC cells

(A) Scratch assay results demonstrated that following a 120-second treatment with CAP, FaDu and TU212 cells were imaged at 0, 6, 12, and 24 hours. The migration capacity was evaluated by measuring the distance between the scratch edges at each time point；(B)CAP inhibits the migration ability of FaDu; (C) CAP inhibits the migration ability of TU212; ***P <0.001, ****P <0.0001，ns indicates no statistical difference.All experiments were performed in at least three independent replicates.

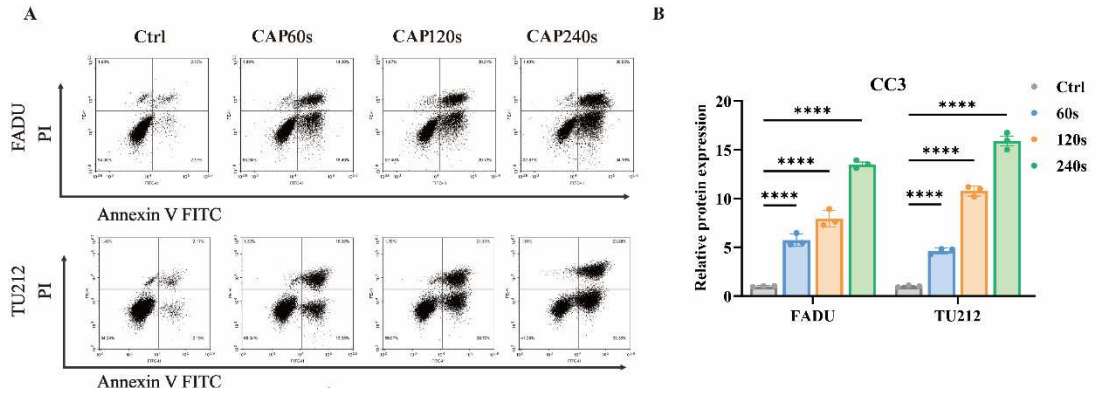


Fig4.CAP induced the apoptosis of HNSCC cells.

(A)Quantitative analysis of apoptosis rates in FaDu and TU212 cells across different treatment groups.(B)Time-dependent increase in apoptosis rates in both cell lines following prolonged CAP exposure. **P <0.01, ***P <0.001, ****P <0.0001. All experiments were performed in at least three independent replicates.

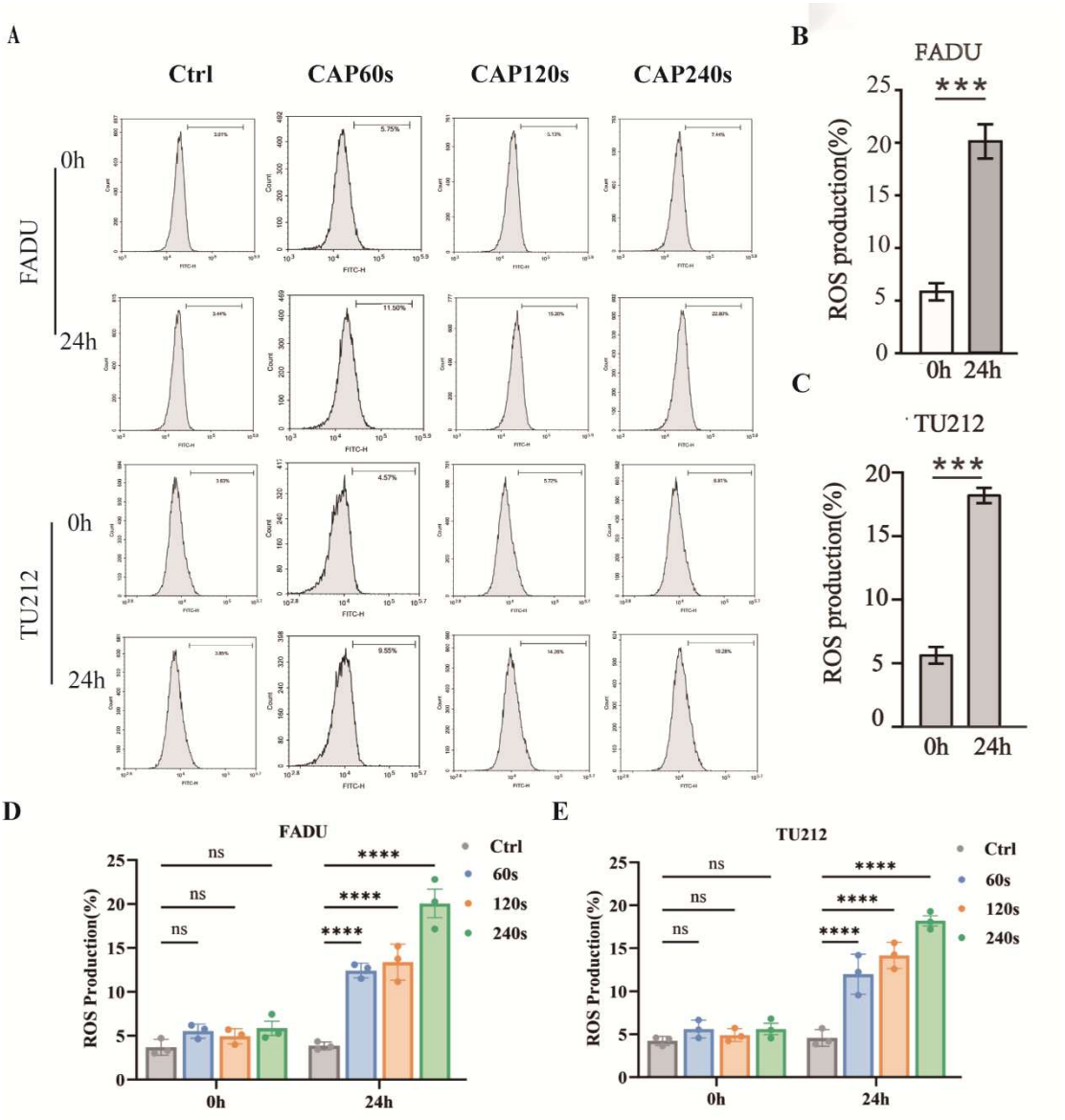


Fig5.CAP Promotes Time-Dependent Intracellular ROS Accumulation in HNSCC Cells after 24 hours.

(A) Quantitative assessment of intracellular ROS levels in FaDu and TU212 cells across four treatment groups (Control, CAP60s, CAP120s, and CAP240s) at 0h and 24h post-CAP treatment. (B–C)flow cytometry plots demonstrating a marked increase in intracellular ROS accumulation in the CAP120s group after 24h of incubation compared to baseline. (D–E)Comparative analysis revealing no significant differences in basal ROS levels among all groups immediately after treatment(0h); however, a statistically significant divergence in ROS accumulation was observed between groups following 24h of incubation, indicating a time-dependent oxidative stress

response. *** p <0.001,**** p < 0.0001,ns， no significance。All experiments were performed in at least three independent replicates.

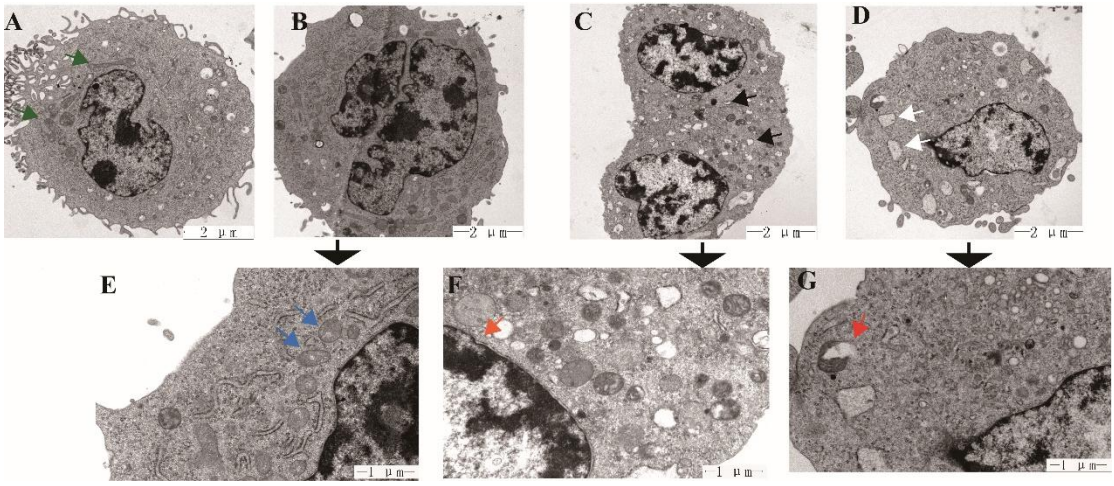


Fig6. Morphology Alterations were observed in FADU cells of the CAP treatment group by TEM.

(A)chromatin was evenly distributed, rich in microvilli and mitochondrial cristae(green arrow) were clearly visible in contral(no treated);(B)After CAP treatment for 60s, Mitochondrial swelling accompanied by the loss of cristae (blue arrow), and chromatin condensed and marginated, indicating early apoptosis;

(C)significant nuclear shrinkage was observed After CAP treatment for 120s, accompanied by mitochondrial swelling and intensified chromatin margination,Endoplasmic reticulum dilation(black arrow) and ribosome detachment were also evident; (D)After CAP treatment for 240s, the nucleus was distinctly shrunken, endoplasmic reticulum vacuolization (white arrows),and mitochondria displayed rupture(red arrow).

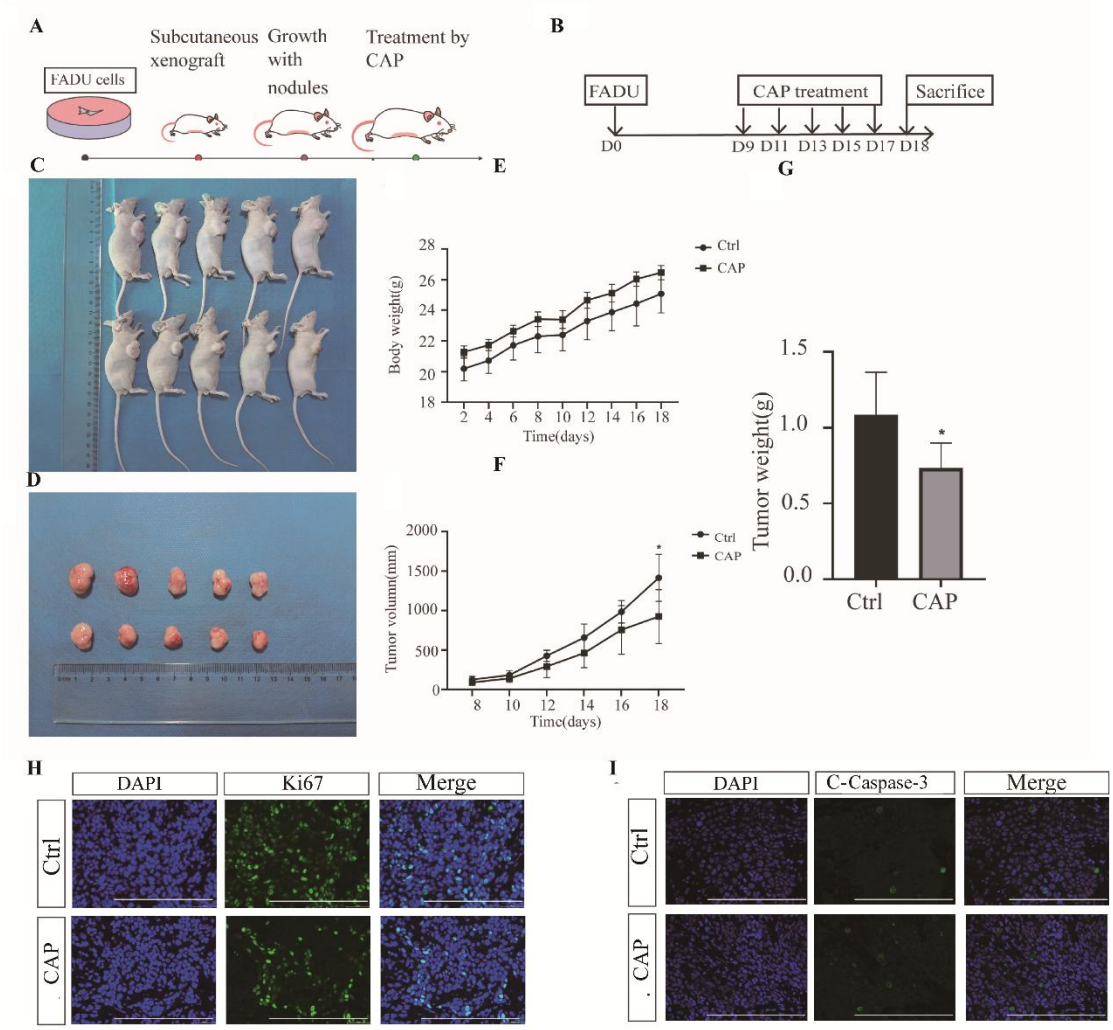


Fig7.Cap treatment inhibited tumor growth in-vivo

(A-B)Schematic diagram of the *in-vivo* experiment.(C-D) State of tumor growth in the human HNSCC cells. The FaDu (4×10^6) cells were injected into nude mice (n = 5).(E)Changes in mice body weight with time.(F) Changes in tumor volume with time.(G)Comparison of tumor weight between the experimental and control group. *P <0.05.(H-I)Immunofluorescence staining of tumor tissues was performed for the detection of Ki67 and C-Caspase3(Scale bar: 20 μm)

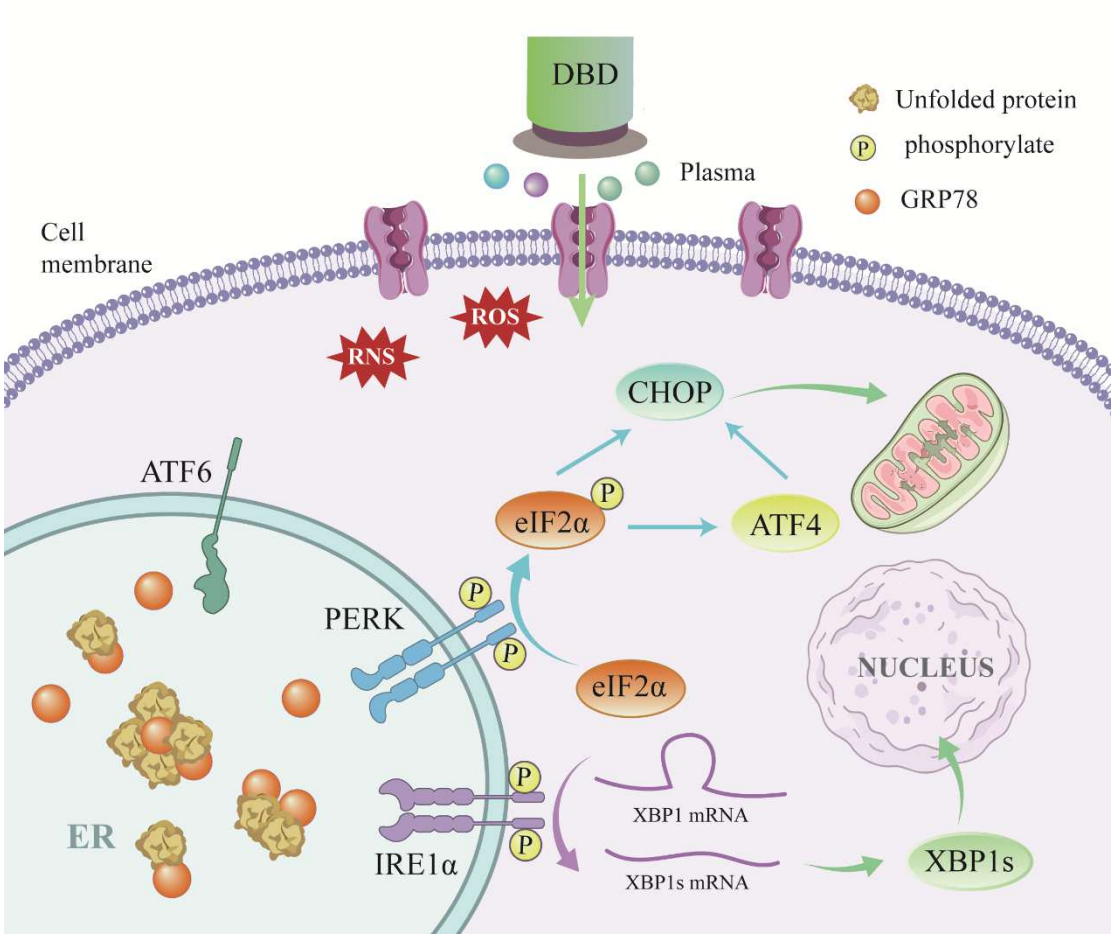


Fig8.Schematic diagram illustrating the activation of endoplasmic reticulum stress through ROS and RNS by CAP