Hyperbaric oxygen combined with 5-aminolevulinic acid photodynamic therapy inhibited human squamous cell proliferation

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Abstract

The photodynamic therapy (PDT) depends on the presence of molecular oxygen. Thus, the efficiency of PDT is limited in anoxic regions of tumor tissue and vascular shutdown. It is reported the use of hyperbaric oxygen (HBO) may enhance the efficiency of PDT. However, there are rarely studies about utilizing HBO plus PDT for treatment with human squamous cell carcinoma (SCC). Therefore, this study aimed to investigate and compare the therapeutic effect of combined therapy and PDT alone treatment. Multiple cellular and molecular biology techniques were used in the current study such as CCK-8, western blotting, flow cytometry, MDC staining and immunofluorescence assay. The results of combination index indicated that HBO combination with PDT synergistically inhibited A431 cells proliferation in vitro. In addition, we found that HBO significantly enhanced PDT-induced cell apoptosis via increasing the active caspase-3, active caspase-9, Apaf-1 and Bax levels and down-regulating Bcl-2. Meanwhile, the result of MDC and immunofluorescence assay confirmed that HBO increased PDT-induced autophagosome formation in A431 cells. Interestingly, autophagy inhibitor 3-methyladenine (3-MA) further increased combination-induced cell apoptosis by increasing the levels of active-caspase 9 and Apaf-1. Our results showed that HBO combined with PDT markedly induced A431 cells apoptosis and autophagy. Nevertheless, autophagy play a pro-survival role against apoptosis. Thus, HBO combination with PDT may constitute a promising approach to treat human squamous cell carcinoma in the future.

Keywords: human squamous cell carcinoma, 5-aminoleinic acid photodynamic therapy, hyperbaric oxygen, apoptosis, autophagy
**Background**

Squamous cell carcinoma (SCC) is a malignant tumor, which derive from keratinocytes arising in the epidermis with the high degree of invasion and destructiveness (1). SCC typically accounts for 20% of non-melanoma skin cancer (NMSC), and is the most common skin cancers (2). The immunosuppressed, xeroderma pigmentosum, and sunburn easily people, who are most susceptible to SCC (3-6). The dangerous environmental factor for SCC is chronic sun exposure, such that the people who work indoors are lower risk than the people who work outdoors (7). The most usual part of SCC is the head and neck (1), which are known as head and neck squamous cell carcinoma (HNSCC) (8). HNSCC is the most common malignant tumor type in the upper aerodigestive tract around the whole world (9).

Over the past 50 years, surgical resection, combination of radiation and chemotherapy are the standard therapy for SCC, while the survival rates were very low always (10). Nowadays, since surgical excision always result in unsightly and hypertrophic scars, photodynamic therapy (PDT) is being using in dermatologic oncology (11). Because of PDT treated NMSCs with the nonscarring and repeatable treatment alternative, particularly for the treatment of carcinomas and precancers (11). A combination treatment was used to kill cancer cells, include photosensitizer (PS) and light in the presence of oxygen (12,13).

5-aminolevulinic acid photodynamic therapy (ALA-PDT) is a photodynamic therapy method of using 5-aminolevulinic acid (ALA) act as photosensitizer. ALA is precursors of protoporphyrin IX (PpIX), which could convert to an intracellular photosensitizer (PpIX) (14-17). The anti-cancer mechanism of ALA-PDT was due to the production of reactive oxygen species (ROS) by PpIX exposure in light (11-13). So far, ALA-PDT is quickly being adopted as a preferred method in dermatologic oncology (14-17). However, the oxygen is a crucial factor in the photochemical reaction. Previous studies have shown that hypoxic cells are less impacted by photosensitizer and light (18-20). Therefore, in order to improve the effectiveness of ALA-PDT, using of
hyperbaric oxygen (HBO) to increase the concentration of oxygen in hypoxic tissue is feasible (21). Nevertheless, there are rarely reports about the SCC treated by HBO combined with ALA-PDT. In this study, we aimed to investigate and compare the therapeutic effect of combined therapy and PDT alone treatment.

Material and methods

Cell culture

Human squamous cell carcinoma cell line A431 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂. A431 cells were seeded into 96-well plate at a concentration of 5×10³ cells/well and incubated overnight. The cells were treated with HBO (0.25 MPa; 98% O₂ + 2% CO₂) for 1 h daily before ALA treatments. ALA was purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA, #A3785). Subsequently, ALA was added into the medium for 48 h at 37°C with 5% CO₂. Meanwhile, the cells were treated with red light using a Phototherapeutics Paterson BL1000A lamp (630 nm ± 15 nm, total dose = 5 J/cm²) from the underside of the plate for 8 h per day (22). The fresh medium with ALA was replaced every 24 h. Experiment groups: control group, HBO alone treatment, ALA-PDT alone treatment and HBO combined with ALA-PDT.

CCK-8 assay of cell viability

Human squamous cell carcinoma cells A431 (5×10³ cells/well) were treated with ALA at different concentrations (0, 0.1, 0.3, 1, 3 μM) for 48 h. Cell viability was measured with Cell Counting Kit-8 (CCK8, Beyotime, Shanghai, China) according to the manufacturer's protocols. The absorbance was determined at 450 nm using a Thermo Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometric analysis of cell apoptosis

A431 cells were cultured overnight in 6-well plates, then cells were treated with
HBO (0.25 MPa; 98% O₂ + 2% CO₂) for 1 h daily, then they were treated with 1 μM ALA and/or 3-MA for 48 h. The cells were not treated with HBO and ALA as control. Afterwards, adherent cells were stained with 10 μL Annexin V and 5 μL propidium iodide (PI) for 15 min at room temperature in the dark and were assessed by means of flow cytometry, according to the manufacturer’s protocols (Thermo Fisher Scientific, Waltham, MA, USA), and measured by FCM flow cytometer (BD Bioscience, San Jose, CA, USA).

**Western blot analysis**

A431 cells were cultured overnight in 6-well plates, cells were treated with HBO (0.25 MPa; 98% O₂ + 2% CO₂) for 1 h daily, then they were treated with 1 μM ALA and/or 3-MA for 48 h. The harvested cells were collected in cell lysis buffer (Beyotime), and the cell lysates were centrifuged at 12,000 rpm for 5 min at 4°C. The protein content in the supernatant was measured using the Bradford Protein Assay Kit (Beyotime, Shanghai, China). The samples were added into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transported onto polyvinylidene fluoride membranes (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) in 2 h. The membranes were blocked with 5% skim milk in TBST for 1 h at room temperature. The blocked PVDF membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies were purchased from Abcam (Cambridge, MA, USA): anti-Bax (Abcam ab32503), anti-Bcl-2 (Abcam ab32124) (1:1000), anti-active caspase 9 (Abcam ab32539), anti-active caspase 3 (Abcam ab2302), anti-Apaf-1 (Abcam ab2001), anti-β-actin (Abcam ab8227). Then the PVDF membrane was incubated with a HRP-conjugated anti-rabbit secondary antibody for 1 h at room temperature. The reactive signals were visualized using the ECL reagent (Santa Cruz Biotechnology). β-actin was used as inner control.

**Monodansylcadaverine (MDC) staining.**

A431 cells were cultured overnight in 6-well plates, cells were treated with HBO (0.25 MPa; 98% O₂ + 2% CO₂) for 1 h daily, then they were treated with 1 μM ALA and/or 3-MA for 48 h. Then the cells were stained with a 0.05 mM final concentration
of (50 μmol/L) MDC (Sigma Aldrich, St. Louis, MO, USA, #D4008) at 37°C for 30 min. A431 cells were washed with PBS 3 times to wipe off redundant MDC. Fluorescence of cells were instantly observed with a Hitachi F-2000 fluorescence spectrophotometer (Olympus Corporation, Tokyo, Japan) and counting.

**Immunofluorescence**

A431 cells were cultured overnight in 6-well plates, cells were treated with HBO (0.25 MPa; 98% O₂ + 2% CO₂) for 1 h daily, then they were treated with 1 μM ALA and/or 3-MA for 48 h. The cells were cultured with primary antibodies for DAPI (ab104139) and LC3 (ab48394) at 4°C overnight. Subsequently, cells were incubated with secondary antibodies (Jackson Immuno Research, USA) at 37°C for 1 h. The samples were immediately observed by fluorescence microscope (Olympus CX23 Tokyo, Japan).

**ROS detection**

A431 cells were cultured in 96-well plate and treated with HBO or/and ALA for 48 h. The level of ROS in the cells were detected with ROS detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocol of manufacture.

**Statistical analysis**

Each group were executed at least three independent experiments and all data were expressed as the mean ± SD. The comparison between two groups was analyzed by Student’s t-test. The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Dunnett’s test. P<0.05 or P<0.01 was considered to indicate a statistically significant difference (*P<0.05, ** P<0.01).

**Results**

**HBO combined with ALA-PDT synergistically suppressed A431 cell proliferation via inducing apoptosis**

As shown in Fig. 1A, the cell proliferation was significantly decreased by a concentration gradient (0, 0.3, 1, 3 μM) of ALA treatments, compared with the control.
group (P<0.01). Meanwhile, HBO combined with ALA-PDT further decreased cell viability compared with the ALA-PDT treatment group (P<0.01). Chou-Talalay’s combination index (CI) method was used in quantifying synergism or antagonism (23). The results indicated that HBO and ALA (0.3, 1, 3 µM) combination in vitro exerted synergistic effect, with CI ranged 0.936-0.823. The combination of HBO with 1 µM ALA showed the optimal synergistic effect (Table 1). Therefore, 1 µM ALA was utilized in the following experiences.

Next, the cell apoptosis was detected by flow cytometry. As indicated in Fig. 1B and C, HBO alone treatment had no effect on cell apoptosis. However, 1 µM ALA interference significantly induced cell apoptosis compared with the control group (P<0.01). In addition, the apoptotic rate was further increased in the presence of HBO treatment in A431 cells, compared with the ALA alone treatment (P<0.01, Fig. 1B, C). These data suggested that HBO combined with ALA-PDT synergistically suppressed A431 cell proliferation

**HBO combined with ALA-PDT synergistically up-regulated Bax and active caspase 3 and down-regulated Bcl-2 in A431 cells**

Western blotting was used to detect the apoptosis- related proteins Bax, Bcl-2 and active caspase-3. As shown in Fig. 2A-D, the expression of these proteins had no significant difference between the control group and the HBO alone treatment. However, the levels of Bax and active caspase 3 were dramatically increased, while Bcl-2 were significantly decreased in the ALA- treated group, compared with the control group (P<0.01). Moreover, the expression of these proteins were further increased (Bax, active caspase 3) or decreased (Bcl-2) respectively by combination treatment, compared with the ALA alone treatment (P<0.01, Fig. 2A-D). These data suggested that HBO combined with ALA-PDT synergistically up-regulated proteins Bax and active caspase 3 and down-regulated Bcl-2 in A431 cells.

**HBO combined with ALA-PDT synergistically induced A431 cell autophagy**

We next assessed if HBO combined with ALA-PDT could A431 cells autophagy. The MDC staining data indicated that the autophagosome was increased in A431
cells treated with ALA compared with the control group, meanwhile the autophagosome formation was further enhanced by HBO compared with the ALA-PDT alone treatment. As expected, autophagosome formation were significantly inhibited by 3-MA treatment, compared with the combined therapy group (P<0.01, Fig. 3A, B). Additionally, immunofluorescence data showed the levels of LC3-II were increased by the combined therapy group, which correlate with autophagosome formation due to its association with the autophagosome membrane (Fig.3C, D). These data indicated that HBO combined with ALA-PDT synergistically induced A431 cell autophagy.

**Inhibition of autophagy enhanced combination-induced apoptosis in A431 cells**

The autophagy inhibitor 3-MA was used to investigate the association between the cell autophagy and cell apoptosis induced by ALA-PDT or/and HBO in A431 cells. The data showed the anti-proliferation of combination was further enhanced by 3-MA (Fig. 4A). In addition, inhibition of autophagy enhanced combination-induced apoptosis in A431 cells (Fig. 4B, C). Moreover, the data of ROS detection indicated HBO enhanced ALA-PDT-induced ROS generation in cells, which was further boosted in the presence of 3MA. All these data demonstrated that autophagy play a pro-survival role in A431 cells treated with combined therapy (Fig. 4D).

**Inhibition of autophagy enhanced combination-induced active caspase 9 and Apaf-1 up-regulation in A431 cells**

Western blotting was utilized to detect the expression of apoptosis related proteins active-caspase 9 and Apaf-1. As shown in Fig. 5A-C, the expression of active-caspase 9 and Apaf-1 were up-regulated in ALA-PDT group, compared with control group (P<0.01). In addition, the expression of these two proteins were further increased in the presence of HBO treatment, compared with the ALA treatment group (P<0.01). Moreover, the level of active caspase 9 and Apaf-1 were increased one step further by 3-MA, compared with the combined therapy group (P<0.01). These results demonstrated that inhibition of autophagy enhanced combination-induced active caspase 9 and Apaf-1 up-regulation in A431 cells.
Discussion

PDT performed a cancer treatment modality of non-invasive, which can effectively eradicate malignancies. PDT destroyed malignant cellular targets via absorbing photosensitizer, visible light and oxygen to generate ROS (24). PDT involves the interaction of photosensitizers, light and oxygen (25). PDT was reported to inhibit cancers through different cell death pathways (26). In this study, ALA-PDT significantly suppressed A431 cell proliferation via inducing mitochondria-dependent intrinsic pathway cell apoptosis, which was evidenced with up-regulation of active caspase 3, Bax, active caspase 9 and Aaf1 levels, and down-regulation of Bcl-2. Fantini et al studied that PDT increased cell apoptosis through up-regulating caspase 3 and down-regulating Bcl-2 levels in situ squamous cell carcinoma (27). Chen et al (28) showed that ALA-PDT significantly inhibited cell proliferation and induced apoptosis via down-regulating Bcl-2 and up-regulating Bax levels in esophageal cancer. ALA-PDT also induced cell apoptosis on cervical cancer cells and human oral squamous cell carcinoma (29). Our findings were similar to these reports. Therefore, we proof that ALA-PDT could inhibited A431 cell proliferation via inducing apoptosis.

Autophagy is affirmed as programmed cell death type II (30, 31). In this study, we found that ALA-PDT significantly induced A431 cell autophagy. The results are consistent with previous study that ALA-PDT could increase the autophagic flux in glioblastoma (32). PDT also increased cell autophagy in human SW620 colon carcinoma cells via upregulating the expression of LC3 (33). All these reports further confirm our findings that ALA-PDT could induce A431 cells autophagy.

The presence of molecular oxygen in tumor tissue is important for the effectiveness of PDT. As is well known, HBO could increase the availability of oxygen in hypoxic tissue. Chou-Talalay’s CI method was used in quantifying synergism or antagonism, where CI<1, =1, and >1 indicates synergism, additive effect and antagonism, respectively (34). Our results indicated that HBO combined with ALA-PDT exerted synergistic anti-proliferation effect on A431 cells in vitro, which might due to an enhancement of apoptosis. In addition, we confirmed that the effect of the combined
therapy group was much better than the ALA-PDT alone treatment group. Moreover, we found HBO enhanced ALA-PDT-induced ROS generation in A431 cells, which was further boosted in the presence of 3MA. All these data demonstrated that autophagy play a pro-survival role in A431 cells treated with HBO plus ALA-PDT via inhibition of ROS generation. More and more evidences has indicated that basal autophagy plays a role of protector against ROS-induced cytotoxicity (35,36). Our data were consistent with these finding. Therefore, induction of autophagy may exhibit a protective effect against the cytotoxic actions in cancer cells, while inhibition of autophagy enhanced cytotoxicity.

In addition, our findings were consistent with previous study that combination treatment improved the efficiency of PDT by increasing the degree of tumor cell necrosis in tumor-bearing nude mice (37). The key to increase cytotoxicity in A431 cells was used HBO, which increased the levels of molecular oxygen in tumor tissues (24,37).

In the current study, we found that HBO-assisted ALA-PDT and 3-MA led to marked cell growth inhibition, ROS generation and increase of apoptosis. These results indicated that inhibition of autophagy enhanced combination-induced apoptosis in A431 cells. This result was consistent with study from Mee et al, who reported that Pa (a kind of Photosensitizer)-PDT exerted anti-tumor effects by inducing apoptosis and autophagy, and autophagy inhibition enhances Pa-PDT-mediated apoptosis in human oral squamous carcinoma cells (25).

Conclusions

Our results showed that HBO combined with PDT markedly induced ROS generation, apoptosis and autophagy in A431 cells. Nevertheless, autophagy play a pro-survival role against apoptosis. Thus, HBO combination with PDT may constitute a promising approach to treat human squamous cell carcinoma in the future.

Conflicts of interest

The authors declare no conflict of interest.
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Figure 1. HBO combined with ALA-PDT synergistically suppressed A431 cell proliferation via inducing apoptosis. (A) Cell viability was determined using CCK-8 assay in A431 cells treated with HBO and/or ALA (0, 0.1, 0.3, 1, 3 μM) for 48 h (**P < 0.01 compared with 0 μM ALA group, ## P < 0.01 compared with ALA alone treatment group). (B) A431 cells were exposed to HBO and/or 1 μM ALA for 48 h. Apoptotic cells were observed with Annexin V and PI double staining. (C) The apoptosis cell rates were calculated (**P < 0.01).
Figure 2. HBO combined with ALA-PDT synergistically up-regulated Bax and active caspase 3 and down-regulated Bcl-2 in A431 cells. A431 cells were pre-treated with HBO 1 h daily, then exposed to 1 μM ALA for 48 h. (A) Expressions of active caspase-3, Bax, Bcl-2 analyzed by western blotting in A431 cells after 48 h. (B) Bax relative expression was quantified by normalizing to β-actin (**P < 0.01). (C) Bcl-2 relative expression was quantified by normalizing to β-actin (**P < 0.01). (D) Active caspase-3 relative expression was quantified by normalizing to β-actin (**P < 0.01).
Figure 3. HBO combined with ALA-PDT synergistically induced A431 cell autophagy. A431 cells were pre-treated with HBO 1 h daily, then exposed to 1 μM ALA and/or 5 mM 3MA for 48 h. (A) MDC staining was used to observe autophagosomes formatting (×200 magnification). (B) The number of autophagosomes in cells was counted (**P < 0.01). (C) Images of the A431 cells stained with LC3 and DAPI. (D) The number of autophagosomes in cells was counted (**P < 0.01).
Figure 4. Inhibition of autophagy enhanced combination-induced apoptosis in A431 cells. A431 cells were pre-treated with HBO 1 h daily, then exposed to 1 μM ALA and/or 5 mM 3MA for 48 h. (A) Cell viability was measured by CCK-8 assay (*P < 0.05, **P < 0.01). (B) Cell apoptotic were observed with Annexin V/PI assay. (C) The apoptosis cell rates were calculated (*P < 0.05, **P < 0.01). (D) The relative ROS level in each group was detected with ROS detection kit (**P < 0.01).
Figure 5. Inhibition of autophagy enhanced combination-induced active caspase 9 and Apaf-1 up-regulation in A431 cells. A431 cells were pre-treated with HBO 1 h daily, then exposed to 1 μM ALA and/or 5 mM 3MA for 48 h. (A) Expressions of active caspase 9 and Apaf-1 analyzed by western blotting after 48 h culture in A431 cells. (B) Active caspase-9 relative expression was quantified by normalizing to β-actin. (E) Active Apaf-1 relative expression was quantified by normalizing to β-actin (**P < 0.01).
Table 1. The CI value evaluation of HBO plus ALA using the method of Chou & Talalay.

| Combination strategy | CI values |
|----------------------|-----------|
| HBO + 0.1 µM ALA     | 1.023     |
| HBO + 0.3 µM ALA     | 0.936     |
| HBO + 1 µM ALA       | 0.823     |
| HBO + 3 µM ALA       | 0.867     |

Combination index (CI) was calculated from the CI equation algorithms using CompuSyn software. CI=1, <1 and >1 indicates additive effect, synergism and antagonism, respectively.