Effects of the combination of hyperbaric oxygen and 5-fluorouracil on proliferation and metastasis of human nasopharyngeal carcinoma CNE-2Z cells

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ABSTRACT

Objective: We investigated the effects of hyperbaric oxygen (HBO₂) and/or 5-fluorouracil (5-FU) on the proliferation and metastasis of human nasopharyngeal carcinoma (NPC) cell line CNE2Z and the underlying mechanisms involved.

Methods: Nasopharyngeal carcinoma (NPC) CNE2Z cells were randomly divided into four groups: Group A: control group; Group B: 5-FU group; Group C: HBO₂ group; Group D: 5-FU plus HBO₂ group. The inhibitory effects on CNE2Z cells proliferation in the four groups after 24, 48, and 72 hours of treatment were measured by MTT-colorimetric method. Transwell chamber assay was performed to determine the effects of HBO₂ and/or 5-FU on the metastasis of CNE2Z cells; Expressions of MMP-9 and VEGF in CNE2Z cells were detected by immunocytochemical staining.

Result: A significant difference was observed in the inhibitory effects on CNE2Z cell proliferation (OD values) between the 5-FU group (Group B) and the control group (Group A) after 24, 48, and 72 hours of treatment (p<0.01); between the HBO₂ group (group C) and the control group (Group A) after 48 and 72 hours of treatment (p<0.01); and between the HBO₂ plus 5-FU group (Group D) and the control group (Group A) as well as HBO₂ plus 5-FU group (Group D) and the HBO₂ group (Group C) after 24, 48, and 72 hours of treatment (p<0.01). But a significant difference between the HBO₂ plus 5-FU group (Group D) and the 5-FU group (Group B) was observed only after 48 hours of treatment (p=0.030). As for metastasis, as well as MMP-9 and VEGF expression OD values, significant difference was observed between the 5-FU group (Group B) and the control group (Group A) with p<0.05, but not between the HBO₂ group (Group C) and the control group (Group A). Although effects on metastasis as well as MMP-9 and VEGF expression OD values were significantly different between the 5-FU plus HBO group (group D) and group A (p<0.01), no difference was observed between Group D and Group B as well as Group D and Group C.

Conclusions: Simple HBO₂ treatment after 48 and 72 hours could inhibit the proliferation of nasopharyngeal carcinoma CNE2Z cells. The combination of HBO₂ with 5-FU exhibited significant synergism in the suppression of NE2Z cell proliferation only after 48 hours of treatment compared to 5-FU. Simple HBO₂ treatment could not reduce the high expressions of MMP-9 and VEGF and inhibit the metastasis of human NPC CNE2Z cells, and no synergistic effect was observed for the combination of HBO₂ with 5-FU compared to 5-FU alone.
INTRODUCTION
Nasopharyngeal carcinoma (NPC) is a common malignant tumor, belonging to poorly differentiated squamous cell carcinoma. A five-year survival rate in end-stage patients occurs in approximately 50% (1).

A large number of experimental and clinical data have confirmed that hyperbaric oxygen (HBO2) can improve the oxygenation of tumor cells to correct tumor hypoxia (2), enhance sensitivity of tumor cells to radiotherapy and chemotherapy (3), and increase local tumor control rate and the five-year survival rate (4). 5-fluorouracil (5-FU) is a metabolic anticancer chemotherapy drug, commonly used in the combination therapy for nasopharyngeal carcinoma clinically to achieve better results (5). This study aimed to explore the effects of HBO2 and/or 5-FU on the proliferation and metastasis of the human NPC cell line CNE2Z and the underlying mechanisms involved.

MATERIALS AND METHODS
Materials and instruments
Human NPC cell line CNE2Z was provided by Cancer Research Institute of Xiangya Medical School; tetrazolium (MTT) reagent was from Sigma, USA; Dimethyl sulfoxide (DMSO) was from Shanghai Seebio Biotech. Inc; artificial basement membrane gel (Matrigel) was from Becton Dickinson Labware, USA; 5-FU was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd; Rabbit anti-human MMP-9 antibody (ZA-0336) and Rabbit anti-VEGF antibody (ZA-0509) were from ZSGB-BIO, Beijing, China; Transwell chamber was from Corning company; LB2 optical microscope was purchased from Leica; Maikeaodi image acquisition and analysis system was from Motic; and baby hyperbaric oxygen chamber (YLC0.5/1a) was from Wuhan Institute of Ship Design.

Reagents
1. RPMI-1640 culture medium was prepared as follows: Dissolve one bag of powered RPMI 1640 (Gibco, Co) in distilled water and add appropriate amount of NaHCO3 to adjust the pH to 7.2. Dilute the final volume to 1 L and sterilize by pressure filtration. Store the aliquots at -20 °C. To complete this medium for CNE2Z cell culture, add 10% of BSA.
2. PBS solution: Dissolve 8g NaCl, 0.2g KCl, 1.54g NaHPO4·12H2O and 0.2g KH2PO4 in 1L water and adjust the pH to 7.0. Sterilize the aliquots by autoclave and store at 4°C.
3. Substrate mixture: Add one drop (about 50ul) of concentrated buffer to 1.5ml double-distilled water (pH of 7.0), mix well. Then add one drop of concentrated DAB and hydrogen peroxide solution and mix well. The solution is protected from light and used within 30 minutes.

METHODS
Culture of NPC CNE2Z cells
Human nasopharyngeal carcinoma CNE2Z cells were cultured in RPMI-1640 containing 10% FBS at 37°C under 5% CO2. The culture medium was replaced when the color changed to yellow. The cells were subjected to digestion and passage by 0.5% trypsin plus 0.2% EDTA at room temperature when the cells reached confluence.

Grouping
Human nasopharyngeal carcinoma CNE2Z cells were divided into four groups:
1. The control group (Group A): without any treatment;
2. 5-FU group (Group B): CNE2Z cells were treated with certain amount of 5-FU calculated by half inhibitory concentration;
3. HBO2 group (Group C): cells were treated by HBO2 (2.0ATA, 90 minutes, Bid × 1d);
4. 5-FU plus HBO2 group (Group D): CNE-2Z cells were treated with same amount of 5-FU as Group B, followed by HBO2 exposure.

HBO2 Treatment
Human nasopharyngeal carcinoma cells CNE2Z in Groups C and D were placed into a type YLCO/1A baby hyperbaric oxygen chamber. HBO2
treatment was carried out as follows: First, crack washing at an oxygen flow rate of 10 L/minute was performed for five minutes to increase the cabin oxygen concentration to more than 55%; it was followed by compression at a speed of 5–8 L/minute for 15 minutes. The cabin pressure was held when it reached 2.0 ata and the oxygen concentration reached more than 85%. At that point both compression and decompression valves were opened during constant pressure and a small flow of oxygen was continued at a flow rate of 3-5 L/minute to wash the cabin. After 60 minutes of oxygen absorbance at a constant pressure, decompression was performed at a constant speed for 15 minutes.

The total treatment time was 90 minutes and repeated after a four-hour interval (6). In addition, a small amount of CO₂ was added during the whole procedure, together with oxygen, in order to maintain 5% CO₂ in the chamber. Cells of groups A and B were also placed in a YLCO/1A baby hyperbaric oxygen chamber under atmospheric pressure for 90 minutes and then repeated.

50% inhibitory concentration of 5-FU (IC₅₀)
Human nasopharyngeal carcinoma CNE2Z cells were seeded onto 96 well plates at the density of 1 × 10⁴/ml and cultured to 80% confluence. The cells were divided into eight groups, with six wells per group. Group 1 was the control group and Group 2 was blank. The remaining groups were treated with 5-FU at the concentration of 12.5, 2.5, 0.5, 0.1, 0.05, 0.01 mg/ml 5-FU in RPMI1640 complete medium containing 10% FBS for 96 hours. MTT method was performed by measuring the absorbance value (OD value) at 492nm-630nm on a microplate reader.

The rate of cell growth inhibition was calculated according to the formula: [cell growth inhibition rate = (OD value of the control group – OD value of treated group)/(OD value of the control group – a blank Group OD value)]. Half inhibitory concentration (IC₅₀) of 5-FU on CNE2Z cells were further calculated by modified Kou-type method: lgiIC₅₀ = Xm-I (P-3-Pm-Pn)/4, in which Xm:lg maximum dose; I: lg (maximum dose/adjacent dose); P: sum of positive response rate; Pm: the largest positive response rate; Pn: the smallest positive response rate.

MTT reduction assay
Human nasopharyngeal carcinoma CNE-2Z cells at exponential phase of growth were subjected to trypsin digestion to produce single cell suspension. The cells were gradient diluted to about 12,000/ml after counting and added to a 96-well plate with 200µl per well. Each group contained 18 wells and a total of four plates were used. The cells were cultured at 37° C under 5% CO₂ to attach to the plate.

The cells were subjected to treatment according to Group A, B, C and D for 24, 48 and 72 hours and incubated at 37° C with 5% CO₂ for 24 hours. A 20µl 5g/L MTT solution was added to each well and incubated for another four hours. The culture medium was discarded by reversing the plate, and 200µl DMSO was added to each well.

After 10 minutes of incubation at 37° C, absorbance value (OD value) at 492nm-630nm wavelength was measured on a microplate reader. Incubation times (24, 48 or 72 hours) that showed inhibitory effects for 5-FU and/or HBO₂ treatments were selected for the following experiments.

Invasion test
Matrigel was diluted to 5µg/µl with RPMI1640 and 100µl of the diluted matrigel was added to the top chamber of a Transwell, followed by air-drying at 4° C overnight. A 100µl (1×10⁵/ml) nasopharyngeal carcinoma cell suspension in RPMI1640 containing 1% BSA was added to the top chamber of the Transwell. A 600µl RPMI 1640 medium containing 5% FBS was added to a 24-well plate, and the transwell chamber was immersed in the 24-well plate in a conditioned medium, cultured at 37° C, 5% CO₂ for 24 hours.

The transwell was removed from the 24-well plate and washed with PBS. The cells on the upper membrane were removed by cotton swab. The membrane was processed by hematoxylin for three minutes, eosin staining for 10 seconds, and digestion in warm water. The cells that did not pass through the membrane were wiped by cotton swab, and cells
passing through the membrane were counted under a 400x microscope. Each membrane was counted at five different fields, and the counts were averaged.

**Migration test**

100μl (1 × 106/ml) nasopharyngeal carcinoma cells after treatment in RPMI1640 medium containing 1% BSA was added to the top chamber of the Transwell. A 600μl RPMI1640 medium containing 2.5% FBS was added to a 24-well plate and the transwell chamber was immersed in a 24-well plate in a conditioned medium, cultured at 37°C under 5% CO₂ for 24 hours.

The transwell was removed from the 24-well plate and washed with PBS. The cells on the upper membrane were removed by cotton swab. The membrane was processed by hematoxylin for three minutes, eosin staining for 10 seconds, and digestion in warm water. The cells that did not pass through the membrane were wiped by cotton swab, and cells passing through the membrane were counted under a 400x microscope. Each membrane was counted at five different fields and the counts were averaged.

**Immunocytochemical staining**

Human nasopharyngeal carcinoma CNE2Z cells were seeded onto a six-well plate with built-in cover slip and washed with cold PBS 3 × 5 minutes after attachment, fixed with pre-cooled formaldehyde-acetone fixative for 10 minutes, incubated in 3% H₂O₂ deionized water for 10 minutes, washed with cold PBS (3 × 5 minutes), added normal goat serum solution, and incubated at room temperature for 15 minutes.

After incubation, excess serum was discarded and a rabbit anti-human antibody solution was added. After incubation in a refrigerator at 4°C overnight, a biotinylated secondary antibody working solution was added and incubated at 37°C for 15 minutes, followed by horseradish enzyme-linked streptavidin working solution and incubated for 15 minutes at 37°C. After DAB treatment for 10 ~ 15 minutes (control of color under a microscope), the plates were washed with water and lightly stained with hematoxylin, washed by tap water for 15 minutes, progressively dehydrated, and sealed by glycerol after air-drying. Observation under the optical microscope and image capture indicated that VEGF-positive staining were brown-yellow or brown fine particles, expressed mainly in cell membrane and cytoplasm. MMP-9 positive staining was also brown-yellow or brown fine particles, expressed mainly in cytoplasm and cell membrane. MMP-9 positive staining was divided into four grades according to the signal strength level: egg-yellow as weakly positive, brown-yellow as positive; brown color as strong positive; and colorless as negative.

Five regions were selected for each group, including top, middle, lower, left, and right; 500 cells were counted. Average optical density value of each group was obtained through Maikeaodi image acquisition and analysis system and then compared.

**Statistics analysis**

All data analysis was performed using statistical software SPSS11.0 and Excel 7.0. Data were expressed as mean ± standard deviation (x ± s). A t-Test was performed for comparison between two groups, and one-way ANOVA was used for multiple-group comparison, with post-hoc comparisons by LSD test if the variances were equal, or Tamhane’s T2 method if the variances were unequal. A value of \( p<0.05 \) was considered statistically significant.

**RESULTS**

50% inhibitory concentration of 5-FU (IC50)

50% inhibitory concentration of 5-FU was calculated to be 0.25 mg/ml (Table 1, facing page).

**Growth inhibiting by MTT reduction assay**

A significant difference was observed for the inhibition of nasopharyngeal carcinoma cell proliferation (OD values) among the four groups (F=83.703, \( p<0.01 \), F=60.344, \( p<0.01 \), F=221.959, \( p<0.01 \)). Of these, the simple HBO₂ group (Group C) after 48 and 72 hours of treatment showed a significant difference from Group A (\( p<0.01 \), \( P=0.001 \)); the simple 5-FU group (Group B) treatment for 24, 48 and 72 hours showed
Invasion and migration

Nasopharyngeal carcinoma cell invasion and migration were significantly different among the four groups (F=5.319, p<0.01, F=5.671, p<0.01). Among these, a significant difference was observed between Group B and A (p=0.002, p=0.001), but not between Group C and A (p=0.107, p=0.068); there was a significant difference between Group D and Group A (p=0.001, but no significant difference was observed between Group D and B as well as C (p=0.824, p=0.84, p=0.066, p=0.079) (Table 3, Page 146).

### TABLE 1 – 50% inhibitory concentration of 5-FU (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Optical density</th>
<th>Inhibition ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.4547±0.063</td>
<td></td>
</tr>
<tr>
<td>Blank group</td>
<td>0.1795±0.017</td>
<td></td>
</tr>
<tr>
<td>5-FU(12.5mg/ml) group</td>
<td>0.2323±0.014</td>
<td>0.81</td>
</tr>
<tr>
<td>5-FU(2.5mg/ml) group</td>
<td>0.2947±0.013</td>
<td>0.58</td>
</tr>
<tr>
<td>5-FU(0.5mg/ml) group</td>
<td>0.3073±0.018</td>
<td>0.54</td>
</tr>
<tr>
<td>5-FU(0.1mg/ml) group</td>
<td>0.3247±0.016</td>
<td>0.47</td>
</tr>
<tr>
<td>5-FU(0.05mg/ml) group</td>
<td>0.3620±0.021</td>
<td>0.34</td>
</tr>
<tr>
<td>5-FU(0.01mg/ml) group</td>
<td>0.3908±0.031</td>
<td>0.23</td>
</tr>
</tbody>
</table>

In Table 1, 50% inhibitory concentration of 5-FU was calculated to be 0.25 mg/ml.

In Table 2, a significant difference from Group A (p<0.01). The HBO₂ plus 5-FU group (Group D) after 24, 48 and 72 hours of treatment showed a significant difference from the control Group A and the simple HBO₂ group (Group C) (p<0.01), while only significantly different from the 5-FU group (Group B) after 48 hours of treatment (p=0.030). Thus, the 5-FU and/or HBO₂ groups showed inhibitory effects on nasopharyngeal carcinoma cell proliferation after 48 hours for four group treatments. The following experiments were performed for 48 hours (Table 2, below).

### TABLE 2 – Comparison of growth inhibiting in groups (x ± s)

<table>
<thead>
<tr>
<th>OD</th>
<th>Groups</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A group</td>
<td>0.5915±0.025^{abc}</td>
<td>0.7435±0.067^{abc}</td>
<td>1.0933±0.091^{abc}</td>
</tr>
<tr>
<td></td>
<td>B group</td>
<td>0.3530±0.018^{de}</td>
<td>0.4085±0.012^{de}</td>
<td>0.2843±0.021^{de}</td>
</tr>
<tr>
<td></td>
<td>C group</td>
<td>0.5528±0.027^{f}</td>
<td>0.5710±0.052^{f}</td>
<td>0.9060±0.063^{f}</td>
</tr>
<tr>
<td></td>
<td>D group</td>
<td>0.3698±0.035</td>
<td>0.3258±0.041</td>
<td>0.2815±0.014</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>F=83.703, P&lt;0.01</td>
<td>F=60.344, P&lt;0.01</td>
<td>F=221.959, P&lt;0.01</td>
</tr>
</tbody>
</table>

- a compared with group B: p=0.000, p=0.000, p=0.000
- b compared with group C: p=0.064, p=0.000, p=0.001
- c compared with group D: p=0.000, p=0.000, p=0.000
- d compared with group C: p=0.000, p=0.000, p=0.000
- e compared with group D: p=0.395, p=0.030, p=0.946
- f compared with group D: p=0.000, p=0.000, p=0.000
TABLE 3 – Comparison of invasion and migration and expression of MMP-9 and VEGF in four groups (x ± s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Invasion(numbers)</th>
<th>Migration(number)</th>
<th>MMP-9</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A group</td>
<td>91.50±10.89abc</td>
<td>118.15±17.24abc</td>
<td>0.4933±0.0889abc</td>
<td>0.4917±0.0538abc</td>
</tr>
<tr>
<td>B group</td>
<td>77.55±14.25de</td>
<td>98.40±18.64de</td>
<td>0.4080±0.0507de</td>
<td>0.3800±0.1191de</td>
</tr>
<tr>
<td>C group</td>
<td>84.55±11.32f</td>
<td>107.50±16.70f</td>
<td>0.4175±0.0556f</td>
<td>0.4183±0.0214f</td>
</tr>
<tr>
<td>D group</td>
<td>76.60±16.58</td>
<td>97.25±20.11</td>
<td>0.3400±0.0337</td>
<td>0.3400±0.0779</td>
</tr>
<tr>
<td>P</td>
<td>F=5.319,P=0.002</td>
<td>F=5.671,P=0.001</td>
<td>F=4.679,P=0.017</td>
<td>F=4.308 P=0.021</td>
</tr>
</tbody>
</table>

a compared with group B: p=0.002, p=0.001, p=0.045, p=0.024
b compared with group C: p=0.107, p=0.068, p=0.089, p=0.087
c compared with group D: p=0.001, p=0.001, p=0.002, p=0.004
d compared with group C: p=0.104, p=0.118, p=0.829, p=0.406
e compared with group D: p=0.824, p=0.842, p=0.137, p=0.428
f compared with group D: p=0.066, p=0.079, p=0.110, p=0.101

Expression of MMP-9 and VEGF
Positive staining was significant and expression was higher for MMP-9 and VEGF in nasopharyngeal carcinoma cells. MMP-9 and VEGF were mostly weakly positive or positively expressed in Groups B, C and D, with egg-yellow or brown granules distributed in the cell cytoplasm and membrane; MMP-9 and VEGF in nasopharyngeal carcinoma cells were mostly positive or strong positively expressed in Group A, with brown-yellow or brown particles distributed in the membrane and cytoplasm of cancer cells (Figure 1 and Figure 2, below).

**FIGURE 1**

![Figure 1](http://archive.rubicon-foundation.org)

Figure 1 – SP immunocytochemical staining of MMP-9 (×400). A: control group; B: 5-FU group; C: HBO₂ group; D: combination of 5-FU and HBO₂ group.

**FIGURE 2**

![Figure 2](http://archive.rubicon-foundation.org)

Figure 2 – SP immunocytochemical staining of VEGF (×400). A: control group; B: 5-FU group; C: HBO₂ group; D: combination of 5-FU and HBO₂ group.
3.5 Optical density of MMP-9 and VEGF
A significant difference was observed for MMP-9 and VEGF expression (OD values) in nasopharyngeal carcinoma cells among the four groups (F=4.679; F=4.308, p <0.05). Among these, a significant difference was observed between Group B and A (Pm=0.045, Pv=0.024); but not between Group C and Group A (p=0.089; p=0.087). There was a significant difference between Group D and Group A (p=0.002, p=0.004), but no significant difference was observed between Group D and B as well as C (p=0.137; p=0.428; p=0.110; p=0.101) (Table 3).

DISCUSSION
Nasopharyngeal carcinoma is one of the most common malignant tumors in southern China and Southeast Asia, with poorly differentiated cancer cells as the major pathological patterns. Invasion and metastasis occurs during the early stage. Its prognosis is closely related to cancer proliferation, invasion and potential migration (7). At present, radiation therapy is the most effective treatment method, but the five-year survival rate of some patients at high grade and late stages is only about 50%. Treatment failure is mainly due to uncontrolled local or regional lesions and occurrence of distant metastasis (8).

In recent years, there has been an increasing trend for the treatment of advanced nasopharyngeal carcinoma by chemotherapy. 5-FU is an effective drug for the treatment of nasopharyngeal carcinoma; it is an anti-metabolic chemotherapy drug and plays a major role in the mitotic S phase, affecting DNA synthesis and promoting cancer cell apoptosis (9).

HBO2 therapy has already been widely used in clinic and has good efficacy for the sensitization of chemotherapy and radiotherapy of certain malignant tumors (10) and treatment of its related symptoms and diseases, such as radiation encephalopathy and syndrome after chemotherapy (11). This study explored the effects of HBO2 and/or 5-FU on nasopharyngeal carcinoma CNE-2Z cell proliferation and invasion and the mechanisms involved.

Effects of HBO and/or 5-FU on nasopharyngeal carcinoma CNE-2Z cell proliferation measured by MTT
Uncontrolled cell growth is one of the most basic biological characteristics of cancer. Thus, the inhibition of tumor cell proliferation and promotion of cell death is the basic approach to treat cancer and the basic requirement for anti-cancer therapy (12). In this study, the MTT assay was used to study the inhibitory effect of HBO2 or (and) 5-FU treatment on nasopharyngeal carcinoma CNE-2Z cell proliferation after 24, 48 and 72 hours of treatment.

Table 2 showed that a simple HBO2 treatment (Group C) after 48 and 72 hours significantly inhibited nasopharyngeal carcinoma cell proliferation compared with the control group with p<0.01, suggesting that HBO2 could inhibit nasopharyngeal carcinoma CNE-2Z cell proliferation (13). This finding is consistent with the results reported by McDonald (14). In their study, they observed 40 golden Syrian hamsters with cheek pouch carcinoma induced by 9,10-1,2-dimethyl-benzanthracene. Among these, 20 were treated with 2.8 ATA × 60 minutes of hyperbaric oxygen and 20 were used as control. The results showed that tumor volume was significantly smaller in the HBO2 treatment group than the control group.

Consistently, Table 2 also showed that simple 5-FU (B) treatment for 24, 48 and 72 hours significantly inhibited nasopharyngeal carcinoma cell proliferation compared with the control (p<0.01). The combination of the HBO2 and 5-FU treatment (Group D) after 24, 48 and 72 hours showed significant difference compared with the control (Group A) (p < 0.01), but compared with the 5-FU group (Group D), a significant difference was observed only after 48 hours (p=0.030). This indicated that a combination of HBO2 and 5-FU was more effective only on the inhibition of cell proliferation after 48 hours of treatment, which was due to the synergistic effect.
Analysis of HBO and (or) 5-FU treatment on nasopharyngeal carcinoma CNE-2Z cell invasion and migration by Transwell chamber assay

Invasion and migration is the main biological characteristics of malignant tumors, which is also extremely complex and includes a multistep process (15,16). In this study, the effects of HBO2 and/or 5-FU on nasopharyngeal carcinoma cell invasion and metastasis were analyzed by Transwell chamber method. Table 3 showed that cell numbers that penetrated the filter membranes or pass through Martrigel base gel were lower than the control after simple HBO2 (Group C) treatment, but not statistically significant (p>0.05). The results showed that a simple HBO2 treatment had no effects on the NPC cell migration and degradation of NPC cells.

Table 3 indicated that cell numbers that penetrated the filter membranes or passed through the Martrigel base gel were lower than the control after a simple 5-FU (Group B) treatment, with significant effects (p=0.002, p=0.001). This suggested that 5-FU significantly inhibited the invasion and metastasis of nasopharyngeal carcinoma cells (17).

In addition, although the number of cells penetrating the filter membrane was significantly decreased in the HBO2 plus 5-FU group (Group D) compared to the control group (Group A) (p=0.001), no significant difference was observed between Group D and the 5-FU group (Group B) as well as Group D and the HBO2 group (Group C) with p > 0.05.

Thus, the HBO2 treatment could not inhibit the invasion and metastasis of nasopharyngeal carcinoma cells, and HBO2 plus 5-FU exhibited no further inhibition effects compared to 5-FU, suggesting that HBO2 had no effects on the invasion and metastasis of nasopharyngeal carcinoma cells.

This finding was not consistent with the results reported by Kawasoe Y, et al. (18) that stated: “Concomitant hyperbaric oxygen clearly enhanced the chemotherapeutic effects of carboplatin on lung metastasis in osteosarcoma-bearing mice.” This might be attributed to the different models used. In addition, the HBO2 experiment was performed only twice in our study, while the animal experiment by Kawasoe, et al. was carried out for five weeks.

Effects of HBO and (or) 5-FU treatment on MMP-9 and VEGF expression in nasopharyngeal carcinoma CNE-2Z cells by immunocytochemistry staining

In recent years, studies have shown that matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF) overexpression in many human malignant tumors is closely related to the invasion and metastasis (19,20). Immunohistochemical staining of nasopharyngeal carcinoma pathological tissue MMP-9 protein by Horikawa, et al. (21) showed that although the MMP-9 protein level had nothing to do with the T stage, it correlated significantly with lymph node metastasis. The average score of MMP-9 lymph node metastasis group was 41.5 and 6.0 for the no lymph node metastasis group.

Wakisaka, et al. (22) reported that VEGF levels were significantly higher in the nasopharyngeal tissues of patients with lymph node metastasis nasopharyngeal carcinoma than those without lymph node metastasis. Additionally, VEGF levels were positively correlated with microvessel density in tumor tissue. As shown in the MMP-9 and VEGF expression figures (Figures 1 and 2), positive expression rate of MMP-9 and VEGF was higher in nasopharyngeal carcinoma cell membrane and cytoplasm with darker staining. In this study, average optical density values of MMP-9 and VEGF expression were lower in Group C (simple HBO2 group) than Group A (Table 3), but not statistically significant (p>0.05). This indicated that HBO2 treatment alone could not reduce the high expression of MMP-9 and VEGF (23); thus HBO2 treatment alone had no effects on the metastasis and invasion of nasopharyngeal carcinoma cells, which was consistent with the reports of Feldmeier, et al. (24).

However, average optical density values of MMP-9 and VEGF expression were lower in Group B (simple 5-FU group) compared with Group A with significant effects (Pm=0.045, Pv=0.024), suggesting that 5-FU alone could significantly reduce the levels of highly expressed MMP-9 and VEGF in the nasopharyngeal carcinoma cell line (25). In addition, Table 3 indicated that the number of cells penetrating the filter membrane was significantly
decreased in the HBP plus 5-FU group (Group D) compared to the control group (Group A) \( (p=0.002) \). Though the expression of MMP-9 and VEGF was lower in the combination group (Group D) compared to Group B and Group C, there was no significant difference. This suggested that HBO2 plus 5-FU could not further decrease the expression of MMP-9 and VEGF in nasopharyngeal carcinoma cells. HBO and 5-FU had no synergic effect on the expression of MMP-9 and VEGF and could not further inhibit the metastasis and invasion of nasopharyngeal carcinoma cells compared to 5-FU.

**CONCLUSION**

HBO2 treatment alone after 48 and 72 hours significantly inhibited the proliferation of human nasopharyngeal carcinoma cells, but the combination of HBO2 and 5-FU significantly inhibited only the proliferation of human nasopharyngeal carcinoma cells after 48 hours. HBO2 treatment alone could not significantly reduce the high expressions of MMP-9 and VEGF in the nasopharyngeal carcinoma cells and could not inhibit the invasion and migration of nasopharyngeal carcinoma cells, either. The same effects were observed for the combination of HBO2 with 5-FU.

**REFERENCES**


