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## Effects of carbon dioxide pneumoperitoneum on metastasis of ovarian carcinoma cell line SKOV<sub>3</sub>

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**Abstract:** The long suspicion of the potential harm of carbon dioxide (CO<sub>2</sub>) pneumoperitoneum exists in laparoscopic cancer surgery. For better understanding of this problem, we targeted this study at the effects of CO<sub>2</sub> pneumoperitoneum on the invasive ability of ovarian carcinoma cell line and the possible mechanism within it. To study the effects of CO<sub>2</sub> pneumoperitoneum on carcinoma cell, SKOV<sub>3</sub> cells were divided into 2 groups, respectively exposed to pneumoperitoneal CO<sub>2</sub>-insufflation and normal conditions. To study the possible mechanism, SKOV<sub>3</sub> cells were divided into 3 groups, one was exposed to CO<sub>2</sub> pneumoperitoneum, one to N<sub>2</sub> and the other to normal conditions served as control. The in vitro adhesive and invasive ability of the cells was analyzed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and Boyden filters metastasis model; the expressions of vascular endothelial growth factor C (VEGF-C) and matrix metalloproteinase 9 (MMP9) were determined by reverse transcription polymerase chain reaction (RT-PCR), and Western blot. We found that the adhesive ratio of SKOV<sub>3</sub> cells exposed to CO<sub>2</sub> was significantly higher than that of the control group; cells exposed to CO<sub>2</sub> invaded the matrigel with a greater number ( $P < 0.01$ ); the expression of VEGF-C exposed to both CO<sub>2</sub> and N<sub>2</sub> was significantly increased compared with control group ( $P < 0.05$ ); the MMP9 expression level of CO<sub>2</sub> group was higher than that of N<sub>2</sub> group,  $P < 0.05$ . We concluded that carbon dioxide pneumoperitoneum may improve the adhesive and invasive ability of ovarian carcinoma cell line in vitro and CO<sub>2</sub> can also be an independent factor to stimulate the expression of MMP9.

**Keywords:** carbon dioxide; pneumoperitoneum; ovarian carcinoma; matrix metalloproteinase 9 (MMP9); vascular endothelial growth factor C (VEGF-C)

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### 1 Introduction

Laparoscopic surgery is recognized for its many benefits to the patient, such as a less morbid postoperative period, less discomfort, earlier return of bowel function, shorter hospitalization, and a much quicker return to daily activities. Laparoscopy has been applied to gynecological surgery for many years in diagnostic procedures and operative interventions

of benign processes. Nevertheless, it was reported that carbon dioxide (CO<sub>2</sub>) pneumoperitoneum might change the inner environment of the abdominal cavity, e.g. increasing acidity, and undermining the integrity of peritoneum [1,2]. Thus, whether these changes affect the biological behavior of malignant tumors and whether the application of laparoscopy to malignancy is proper has been a matter of continuing debate.

To clarify the effects of CO<sub>2</sub> pneumoperitoneum on the invasive capability of ovary carcinoma cells SKOV<sub>3</sub> which were cultured respectively in exposure to CO<sub>2</sub>, N<sub>2</sub> and normal environment, applying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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(MTT) assay, reverse transcription polymerase chain reaction (RT-PCR), Western blot, and Boyden filters metastasis model. Consideration of N<sub>2</sub> environment was to explore the possible involvement of hypoxia as a major factor to cause adhesion formation and other biological changes as reported in Refs. [1,3]. We found that CO<sub>2</sub> induces matrix metalloproteinase 9 (MMP9) expression and raises the adhesive and invasive ability of ovarian carcinoma cell line in vitro.

## 2 Materials and methods

### 2.1 Cell lines

Human epithelium ovarian carcinoma cell line SKOV<sub>3</sub> and mouse fibroblast cell line NIH3T3 were obtained from the Ultrasound Lab, Chongqing University of Medical Sciences. The cells were grown in RPMI 1640 medium (Gibco BRL, Life Technologies) supplemented with fetal bovine serum (FBS) of a mass fraction 10% (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and an antibiotic-antimycotic agent containing 100 IU/mL penicillin and 0.1 mg/mL streptomycin. The cells were cultured in flasks at 37 °C in air with a CO<sub>2</sub> mass fraction of 5%. For NIH3T3, when a flask was 90% filled, the culture medium was replaced by only RPMI-1640; and after 24 h, the supernatant fluid was collected to be used as chemotatic factors.

### 2.2 Pneumoperitoneum model

Flasks on which SKOV<sub>3</sub> cells had been seeded 12 h before, were divided into 3 groups, each incubated for 2 h at 37 °C and 0.013 kPa (10 mmHg) in an airtight container respectively insufflated with 100% CO<sub>2</sub>, 100% N<sub>2</sub>, and air of a CO<sub>2</sub> mass fraction 5% as control.

### 2.3 MTT assay

Cells after digested by trypsin of a mass fraction 0.1% were collected and diluted to 200 μL<sup>-1</sup> suspension. The 96-well plates (Corning Costar Corp.) were coated with 5 μg artificial basement membrane Matrigel (Sigma) and treated with bovine serum albumin (BSA) (Hyclone) of a mass fraction 2%.

Altogether 64 samples, grouped in 8, were incubated in air of a 5% CO<sub>2</sub> mass fraction at 37 °C for 30 min, 60 min, 90 min, and 120 min, respectively. The culture medium was then removed and the unattached cells were washed away by phosphate-buffered saline (PBS). Each sample was added with 20 μL MTT and 180 μL RPMI 1640, and incubated in air of a CO<sub>2</sub> mass fraction 5% at 37 °C for 3 h, and then added with 200 μL dimethyl sulfoxide (DMSO) (Chongqing Oriental Agent Company). The absorbance ( $A_C$  for samples exposed to 100% CO<sub>2</sub>,  $A_N$  for those to 100% N<sub>2</sub>, and  $A_0$  for those to air of a CO<sub>2</sub> mass fraction 5%) was measured at 570 nm through a BIO-TEK reader. The adhesive ratios,  $R_C$  and  $R_N$  were defined in terms of absorbance respectively as

$$R_C = \frac{A_C}{A_0} \times 100\%$$

and

$$R_N = \frac{A_N}{A_0} \times 100\%.$$

Each assay was performed in triplicate and the experiments were repeated on at least three separate isolations of SKOV<sub>3</sub> cells.

### 2.4 Boyden chamber cell migration assay

Boyden chamber cell migration assays were performed using Transwells<sup>TM</sup> (Corning Costar Corp.) containing 8 mm-pore polycarbonate membranes. The upper surface of the membrane was coated with 30 μL artificial extracellular matrix Matrigel. The supernatant fluid of NIH3T3 was added to 24-well plates (Corning Costar Corp.) as chemotatic factors. Each well was put in a Boyden chamber seeded with 200 μL SKOV<sub>3</sub> cell suspension of 200 μL<sup>-1</sup>, and then incubated in air of a CO<sub>2</sub> mass fraction 5% at 37 °C for 24 h. Then, the chambers were taken out and the upper surface of the membrane was wiped with a cotton-tip applicator to remove non-migratory cells. The chambers were fixed by 70% alcohol and stained with hematoxylin and eosin (HE). The polycarbonate membranes were carefully taken off, and after dried, mounted respectively onto a slide. The number of migrated cells per microscopic field was counted.

There were 6 samples in each group and the mean of the 6 results was taken as the value of the group. The chemotaxis test by the Boyden chamber was performed similarly, except that no Matrigel coating covered the membrane and the incubation lasted for only 8 h. Each assay was performed in triplicate and the experiments were repeated on at least three separate isolations of SKOV<sub>3</sub> cells.

### 2.5 RT-PCR

After treatment, research groups and control groups were incubated in air of a CO<sub>2</sub> mass fraction 5% at 37 °C for respectively 12 h, 24 h, and 48 h. RNA was extracted according to the manufacturer's instructions of the kit (Tiangen Corp.). Reverse transcription and PCR were conducted according to the manufacturer's instructions of the RT-PCR kit (TaKaRa Corp.) with primers in Table 1. The assay was repeated for 3 times and the value was the mean of triplicate results. Images were acquired and quantitated by SYNGENE Imaging.

### 2.6 Western blot

Western blot analysis was performed as previously described [4]. Briefly, harvested cells were lysed in a cell extraction buffer (Pierce corp.) and the protein sample amount was 20 µg for sodium dodecyl sulphate-polyacrylamide gel electrophoresis; the mass fraction of acrylamide in the gel was 8% for vascular endothelial growth factor C (VEGF-C) and 10% for MMP9, respectively. Separated proteins were blotted to a nitrocellulose (NC) membrane. After blocking with PBS containing 0.1% Tween 20 and 5% skimmed milk powder, the membrane was incubated with goat antihuman VEGF-C, MMP9 and β-actin (Santa Cruz Biotechnology) at 1:500 followed by horseradish peroxidase-conjugated rabbit antigoat IgG. Signals were visualized with an electrochemiluminescence western blotting kit (Beijing Zhongshan). An assay was repeated for 3 times and its value was the mean of 3 triplicate results. Images were acquired and quantitated by SYNGENE Imaging.

Table 1 Primer sequences and base pair sizes

Protein	Primer sequences	Product base pair size
β-actin	5'-GGACTTCGAGCAAGAGATGG-3' 5'-AGCACTGTGTTGGCGTACAG-3'	234
VEGF-C	5'-GGAAAGAAGTTCCACCACCA-3' 5'-AAGAGGCCTTTTGCAGATGA-3'	326
MMP9	5'-CTCATGTACCCTATGTACCG-3' 5'-AGTACTTCCCATCCTTGAAC-3'	367

### 2.7 Statistical analysis

The t-test was used to assess the difference between 2 groups. SNK (student-Newman-Keuls) was used to assess the difference among 3 groups. Data were expressed as mean ± standard deviation and a *P* value less than 0.05 was considered significant.

## 3 Results

### 3.1 Effects of CO<sub>2</sub> pneumoperitoneum on adhesive capability of SKOV<sub>3</sub> cells

The adhesive ratio of SKOV<sub>3</sub> cells increased as

time prolonged. The adhesive ratio of CO<sub>2</sub> pneumoperitoneum treated SKOV<sub>3</sub> cells was significantly higher than that of the control group (Table 2).

Table 2 The adhesive capability of SKOV<sub>3</sub> cells after CO<sub>2</sub> treatment by MTT

Incubation time/min	A <sub>C</sub> /%*	A <sub>0</sub> /%
30	36.38±0.89	28.23±1.09
60	61.42±0.96	47.22±1.02
90	83.65±0.96	72.56±0.95
120	90.36±0.98	81.15±0.85

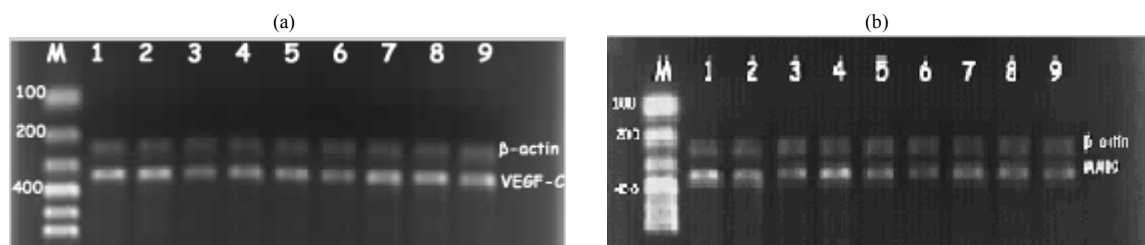
\**P*<0.01 vs. control

### 3.2 Effects of CO<sub>2</sub> pneumoperitoneum on invasiveness of SKOV<sub>3</sub> cells

In the Boyden chamber cell migration assays, the number of migrated cells of a research group,  $N_{mr}$  was significantly ( $P<0.01$ ) larger than that of the control group,  $N_{m0}$ ; the number of treated cells invading the Matrigel,  $N_{ir}$ , was also significantly ( $P<0.01$ ) greater than that of the control group,  $N_{i0}$ , as can be seen in Table 3.

Table 3 The invasiveness of SKOV3 cells after CO<sub>2</sub> treatment by Boyden chamber cell migration assays.

$N_{mr}$	$N_{m0}$	$N_{ir}$	$N_{i0}$
123.26±9.96*	86.65 ±6.15	72.36 ±3.98*	36.12±4.18



Lane M was the Marker; Lanes 1, 2 and 3 were samples of 12 h, Lanes 4, 5, 6 of 24 h and Lanes 7, 8, 9 of 48 h after treated respectively in 100% CO<sub>2</sub>, 100% N<sub>2</sub> and air of a CO<sub>2</sub> mass fraction 5%

Fig. 1 Expressions of (a) VEGF-C mRNA and (b) MMP9 mRNA measured by RT-PCR

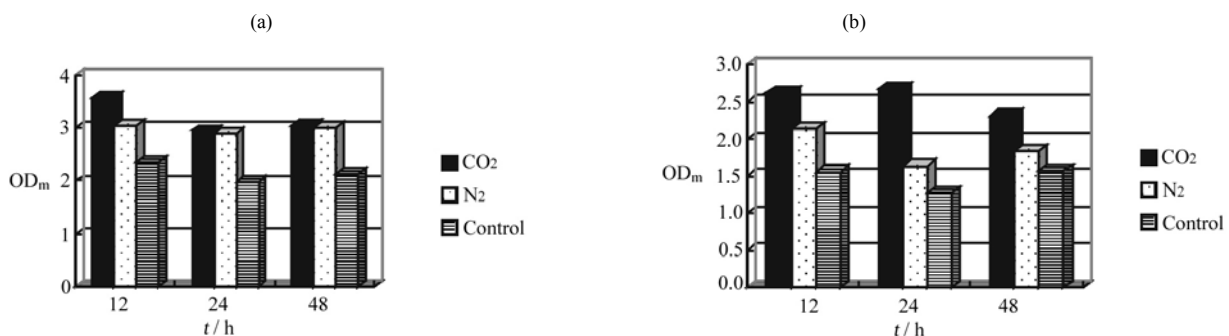


Fig. 2 Mean optical density (OD<sub>m</sub>) of (a) VEGF-C mRNA and (b) MMP9 mRNA in different time ( $t$ ) respectively after CO<sub>2</sub>, N<sub>2</sub> and air (as control) treatments with 100% CO<sub>2</sub>, 100% N<sub>2</sub> and air of a CO<sub>2</sub> mass fraction 5%

### 3.4 Effects of CO<sub>2</sub> pneumoperitoneum on VEGF-C and MMP9 protein expression of SKOV<sub>3</sub> cells

Both CO<sub>2</sub> treatment and N<sub>2</sub> treatment significantly ( $P<0.05$ ) up-regulated VEGF-C protein expression (Fig. 3). The VEGF-C protein expression level of the CO<sub>2</sub> group was significantly ( $P<0.05$ ) higher than the

\* $P<0.01$  vs. control

### 3.3 Effects of CO<sub>2</sub> pneumoperitoneum on VEGF-C and MMP9 mRNA expressions in SKOV<sub>3</sub> cells

The VEGF-C mRNA expression significantly ( $P<0.05$ ) increased after CO<sub>2</sub> or N<sub>2</sub> treatment; VEGF-C mRNA expression level of CO<sub>2</sub> group was a little higher but without statistic significance ( $P>0.05$ ) than that of N<sub>2</sub> group at 24 h or 48 h after treatment (Figs. 1(a) and 2(a)). The MMP9 mRNA expression was significantly enhanced after CO<sub>2</sub> or N<sub>2</sub> treatment,  $P<0.05$ ; MMP9 mRNA expression level of CO<sub>2</sub> group was higher than that of N<sub>2</sub> group,  $P<0.05$  (Fig. 1(b) and Fig. 2 (b)).

N<sub>2</sub> group at 12 h after treatment, and only a little higher without statistic significance ( $P>0.05$ ) at 24 h or 48 h after treatment. The CO<sub>2</sub> treatment and N<sub>2</sub> treatment both significantly ( $P<0.05$ ) increased MMP9 protein expression (Fig. 4), and the MMP9 protein expression level of CO<sub>2</sub> group was also significantly ( $P<0.05$ ) higher than that of N<sub>2</sub> group.

#### 4 Discussion and conclusions

The use of mini-invasive techniques in surgical oncology should be beneficial in terms of the oncological effect on tumor as compared with conventional surgery. However, concerns about the oncological safety of laparoscopic surgeries are increasing,

especially for such phenomena as wound metastases, local recurrences, and intra-abdominal tumor cell dissemination [5].

We investigated the CO<sub>2</sub> pneumoperitoneum effects possibly incurred during a laparoscopic surgery, using SKOV<sub>3</sub> cells.

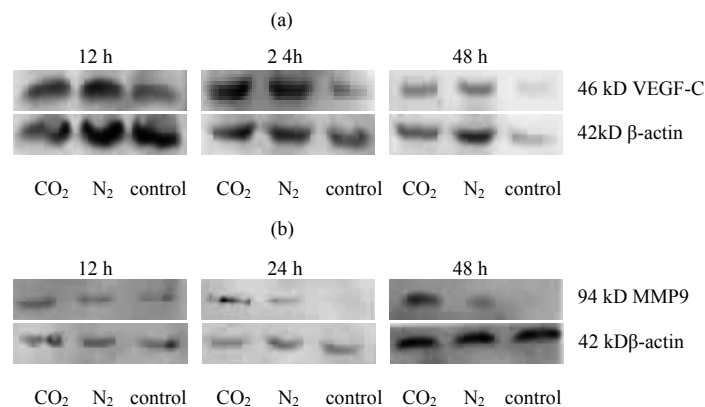


Fig. 3 Protein expression of (a) VEGF-C and (b) VEGF-C measured by Western blot

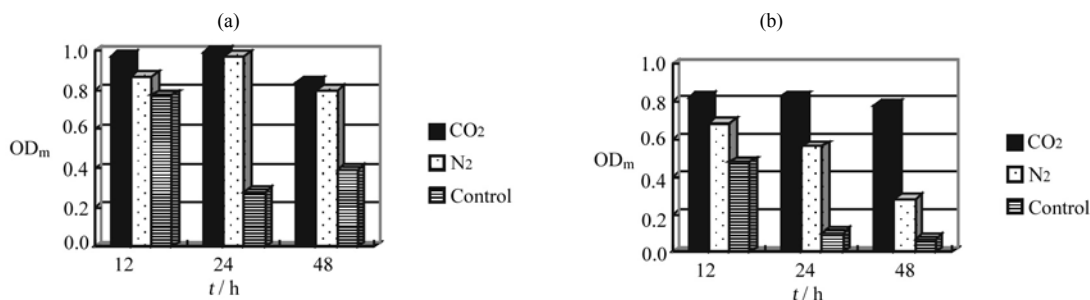


Fig. 4 Mean optical density (OD<sub>m</sub>) of (a) VEGF-C protein and (b) MMP9 protein in different time (*t*) respectively after CO<sub>2</sub>, N<sub>2</sub> and air (as control) treatments with 100% CO<sub>2</sub>, 100% N<sub>2</sub> and air of a CO<sub>2</sub> mass fraction 5%

Our results suggested that the adhesive capability of CO<sub>2</sub> treated SKOV<sub>3</sub> cells was significantly higher than that of the control group, and the adhesive ratio increased as the time after CO<sub>2</sub> treatment passed; the number of cells invading the Matrigel and the number of migrated cells of research group were significantly larger than those of the control groups, respectively. These results indicate that CO<sub>2</sub> pneumoperitoneum increases the invasive ability of SKOV<sub>3</sub> cells.

VEGF-C, as a member of VEGF family, has been recently discovered and characterized to be closely related to a variety of human tumors, including ovarian tumors. VEGF-C expression in cancer cells is higher than in normal tissue. Over expressed VEGF-C results in serious metastatic lesion, and is strongly correlated with clinical stages, retroperitoneal lymph

node metastasis, and lymphangiogenesis in ovarian carcinoma tissues. Inhibiting VEGF-C expression or function has been proved capable of interrupting metastasis [6,7]. The VEGF-C expression up-regulated by CO<sub>2</sub> treatment observed in this study suggested that CO<sub>2</sub> pneumoperitoneum is favorable for the invasiveness of SKOV<sub>3</sub> cells. Moreover, obvious increase in both mRNA expression and protein expression of VEGF-C in the CO<sub>2</sub> group compared with the N<sub>2</sub> group and only the 12-hour subgroup showing statistical significance indicate that besides hypoxia, CO<sub>2</sub> may be an independent factor to promote VEGF-C expression and exacerbate metastasis of SKOV<sub>3</sub> cells.

MMPs play an important role among enzymes in breaking extracellular matrix. MMP-9 may contribute

to the development of ovarian tumors and is strongly associated with tumor cell invasion of ovarian carcinoma cells [8-10]. The effect of CO<sub>2</sub> on MMP9 expression in SKOV<sub>3</sub> turns out to be up-regulation, and hence increases invasiveness of SKOV<sub>3</sub> cells. Both mRNA expression and protein expression of MMP9 in the CO<sub>2</sub> group are significantly higher than those in the N<sub>2</sub> group indicates that hypoxia is only a co-factor of MMP9 up-regulation and CO<sub>2</sub> can be an independent factor to promote MMP9 expression and enhance metastasis of SKOV<sub>3</sub> cells. This is consistent with the implication of VEGF-C expression described above.

A lot of gynecological surgeries including cancer resection are carried out with laparoscopy. Clinical trials have proved that laparoscopic surgery is feasible for gynecological malignant diseases. However, reports on cancer metastasis in the trocar site have been reported and has aroused a debate about whether laparoscopic surgery is suitable for malignant diseases. There is no consensus on whether the incidence of metastasis at a trocar site is higher than that in the wound of an open surgery [4]. The development of solid tumors is a complicated process involving various factors; one cell line and a few factors can not provide conclusive evidence for the efficacy of this type of surgery. Therefore, more clinical trials and further laboratory studies are needed to demonstrate that laparoscopic surgery is indeed a suitable treatment for early malignant diseases.

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