Effects of simulated carbon dioxide and helium pneumoperitoneum on proliferation and apoptosis of gastric cancer cells

Ying-Xue Hao, Hua Zhong, Chao Zhang, Dong-Zu Zeng, Yan Shi, Bo Tang, Pei-Wu Yu

AIM: To investigate the effects of carbon dioxide (CO\textsubscript{2}) and helium insufflation administered at different pressures on the growth and apoptosis of cultured human gastric cancer cells.

METHODS: The gastric cancer cells MKN-45 were exposed to a CO\textsubscript{2} and helium environment maintained at different pressures (0, 5, 10 and 15 mmHg). The cells were exposed to simulated pneumoperitoneum environment for 4 h, and pH of the culture media was measured after it was moved to normal conditions for 0, 2, 4, 6 and 8 h. Proliferation viability of MKN-45 was examined by 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay after it was moved to normal conditions for 0, 2, 4, 6 and 8 h. Proliferation viability in helium group was not obviously different from the control group (P > 0.05). The apoptotic ratio of the cultured cells was markedly higher than that of the control group (P < 0.01) at 10 and 15 mmHg CO\textsubscript{2} insufflation pressure. In helium group, the apoptotic ratio was not obviously different from the control group (P > 0.05).

CONCLUSION: There is no obvious effect in the proliferation and apoptosis of MKN-45 cells under 10 mmHg CO\textsubscript{2} insufflation pressure and helium in any pressure. Fifteen mmHg CO\textsubscript{2} insufflation pressure can inhibit the proliferation of the cells and improve apoptosis.

Key words: Pneumoperitoneum; Gastric cancer cells; Proliferation; Apoptosis

INTRODUCTION

Minimally invasive techniques are increasingly applied in abdominal surgeries\cite{1,2}. In recent years, numerous authors reported an acceptable feasibility of minimally invasive techniques for biopsy and resection of various malignant tumors\cite{3,4}. However, laparoscopic resection for intra-peritoneal malignancies remains controversial. One of the reasons is the concern whether carbon dioxide (CO\textsubscript{2}) pneumoperitoneum can improve cancer cells’ growth\cite{5,6}. There is an ongoing debate about the deleterious effects of CO\textsubscript{2} on tumor cell behavior. Some authors showed an increase in cell proliferation and tumor growth\cite{7,8} and others found beneficial effects of CO\textsubscript{2} exposition in vitro and in animal studies\cite{9,10}.

It is well known that intracellular and extracellular pH in the peritoneum is affected by CO\textsubscript{2} insufflation. And some authors reported that pH in peritoneal cavity may be an important regulator of cell functions, such as adenosine
triphosphate (ATP) production, cell proliferation, and apoptosis[10]. Apart from the acid of peritoneal cavity, whether the direct insult of insufflation pressure could affect the growth of tumor cells is unclear.

Therefore, we focused on the different gases and pressures in simulated pneumoperitoneum, and investigated the proliferative viability of gastric cancer cells and apoptotic ratio in vitro.

**MATERIALS AND METHODS**

**Cell culture**

Human gastric cancer cells (MKN-45; personal gift of Professor F Daiming, Fourth Military Medical University) were cultured in RPMI-1640 (HyClone, USA) culture medium supplemented with 100 g/L fetal bovine serum, penicillin G 100 IU/mL and streptomycin sulfate 100 μg/mL.

**Pneumoperitoneum model in vitro**

To simulate the environment produced during laparoscopic surgery, we designed an in vitro pneumoperitoneum according to Ridgway’s method[11]. We used 100% CO or 100% helium as the insufflation gas-displacement model. Sub-confluent MKN-45 cells which had been plated on 6 cm Petri dishes were placed into modified desiccating chambers. CO or helium insufflation was affected by the connection of a standard surgical insufflator (Stryker, USA) to the chamber. Cells were exposed to a continual pneumoperitoneum for 4 h at 0, 5, 10 and 15 mmHg at 37°C. The pH of the media was examined using an arterial blood gas analyzer (Radiometer ABL 505, Denmark). After the cells were exposed to CO or helium for 4 h, the media was changed and the cells were allowed to grow for 24 h before 3-[4,5Dimethylthiazol-2-yl],5-diphenyltetrazolium bromide or triazolyl blue (MTT) assay or flow cytometry.

**Cell viability**

Cell growth was determined with a spectrophotometric assay[12]. This water-soluble tetrazolium salt was cleaved by the mitochondrion of living cells to an insoluble purple formazan. Optical density readings were measured at 490 nm.

**Percentage of apoptotic cells**

The percentage of apoptotic cells was determined by FITC-labeled Annexin V and PI double staining flow cytometry. The cell growth and apoptosis for each group were compared with those of the control group using one-way analysis of variance (ANOVA). P values less than 0.05 were considered significant.

**RESULTS**

**Influence of pneumoperitoneum on pH of media**

The pH of media in CO₂ and helium group is shown in Tables 1 and 2. When the pressure of CO₂ pneumoperitoneum was 15 mmHg, the pH of media was 6.18. It became normal after 4 h when moved to normal cultured environment. In the helium group, the pH of the media was 8.12 when the pressure was 15 mmHg. Six hours later, it dropped to 7.18 when it was moved to normal cultured environment (Tables 1 and 2).

**MTT assay**

According to MTT chromometry, the proliferative viability of MKN-45 cells was significantly decreased from d 4 to d 7 after it was exposed to simulated CO₂ pneumoperitoneum at 15 mmHg. When the pressure was under 10 mmHg, the cells’ proliferative viability was not obviously different from the control group (P > 0.05). In the helium group, there was no difference between various pressures and control group (P > 0.05), even at 15 mmHg (Tables 3 and 4).

**Percentage of apoptotic cells**

The percentage of apoptotic cells in 10 and 15 mmHg CO₂ groups was significantly higher than control group (P < 0.01). In the helium group, there was no significant difference in the percentage of apoptotic cells under different pressures (P > 0.05). Even the pressure was 15 mmHg, there was no difference from d 4 to d 7 after it was exposed to simulated CO₂ pneumoperitoneum at 15 mmHg. When the pressure was under 10 mmHg, the cells’ proliferative viability was not obviously different from the control group (P > 0.05). In the helium group, there was no difference between various pressures and control group (P > 0.05) (Table 5).

**DISCUSSION**

Several prospective, randomized studies on laparoscopically assisted surgeries for early gastric cancer have demonstrated that the 5-year survival of patients with laparoscopically assisted radical resection of gastric carcinomas was similar to or even higher than that of open surgery[13]. Since March 2004, we have performed 304 cases of laparoscopically assisted surgery.
Table 3  Changes of MKN-45 proliferative viability in CO₂ groups (OD, mean ± SD)

| Groups   | 1      | 2      | 3      | 4      | 5      | 6      | 7      |
|----------|--------|--------|--------|--------|--------|--------|--------|
| Control  | 0.31 ± 0.04 | 0.41 ± 0.02 | 0.55 ± 0.09 | 1.38 ± 0.04 | 1.81 ± 0.09 | 2.33 ± 0.04 | 2.33 ± 0.06 |
| 0 mmHg   | 0.28 ± 0.06 | 0.41 ± 0.04 | 0.56 ± 0.06 | 1.37 ± 0.07 | 1.38 ± 0.02 | 2.38 ± 0.06 | 2.39 ± 0.08 |
| 5 mmHg   | 0.31 ± 0.05 | 0.35 ± 0.05 | 0.56 ± 0.04 | 1.34 ± 0.04 | 1.59 ± 0.07 | 2.50 ± 0.07 | 2.32 ± 0.04 |
| 10 mmHg  | 0.29 ± 0.02 | 0.36 ± 0.04 | 0.55 ± 0.05 | 1.27 ± 0.05 | 1.57 ± 0.14 | 2.54 ± 0.10 | 2.40 ± 0.03 |
| 15 mmHg  | 0.32 ± 0.03 | 0.39 ± 0.05 | 0.47 ± 0.05 | 0.68 ± 0.04b | 0.80 ± 0.04b | 1.16 ± 0.08b | 1.42 ± 0.02b |

*p < 0.01 vs control group; OD: Optical density.

Table 4  Changes of MKN-45 proliferative viability in helium groups (OD, mean ± SD)

| Groups   | 1      | 2      | 3      | 4      | 5      | 6      | 7      |
|----------|--------|--------|--------|--------|--------|--------|--------|
| Control  | 0.29 ± 0.04 | 0.36 ± 0.04 | 0.58 ± 0.03 | 1.22 ± 0.05 | 1.83 ± 0.03 | 2.21 ± 0.04 | 2.62 ± 0.04 |
| 0 mmHg   | 0.30 ± 0.02 | 0.39 ± 0.02 | 0.60 ± 0.04 | 1.23 ± 0.06 | 1.86 ± 0.06 | 2.37 ± 0.05 | 2.64 ± 0.05 |
| 5 mmHg   | 0.30 ± 0.02 | 0.38 ± 0.03 | 0.58 ± 0.03 | 1.27 ± 0.05 | 1.80 ± 0.08 | 2.40 ± 0.33 | 2.75 ± 0.12 |
| 10 mmHg  | 0.31 ± 0.03 | 0.41 ± 0.03 | 0.57 ± 0.05 | 1.25 ± 0.06 | 1.78 ± 0.04 | 2.49 ± 0.26 | 2.71 ± 0.18 |
| 15 mmHg  | 0.33 ± 0.02 | 0.39 ± 0.04 | 0.54 ± 0.09 | 1.24 ± 0.23 | 1.81 ± 0.09 | 2.31 ± 0.20 | 2.73 ± 0.11 |

OD: Optical density.

Table 5  Changes of MKN-45 apoptotic ratio in CO₂ and helium groups (% mean ± SD)

| Groups   | Control | 0 mmHg | 5 mmHg | 10 mmHg | 15 mmHg | F       |
|----------|---------|--------|--------|---------|---------|---------|
| CO₂      | 0.21 ± 0.02 | 0.19 ± 0.04 | 0.29 ± 0.05 | 9.20 ± 0.48 | 11.60 ± 0.95 | 430.09 |
| He       | 0.28 ± 0.04 | 0.27 ± 0.04 | 0.31 ± 0.08 | 0.35 ± 0.11 | 0.37 ± 0.05 | 0.99    |

*p < 0.05 vs control group; CO₂: Carbon dioxide; He: Helium.

assisted gastrectomy, 236 of the cases were advanced gastric cancer. We found no obvious difference between excising tumor with tumor-free margin and dissecting lymph nodes radically[14,15]. However, laparoscopic resection for abdominal malignancy remains controversial, especially for advanced gastric cancer. Among the reasons for this is the concern whether CO₂ pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence[16,17].

The results of experimental studies on the behavior of tumor cells exposed to CO₂ are not conclusive. Numerous authors confirmed a CO₂ associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors using animal models[18-20]. However, other studies showed that CO₂ pneumoperitoneum could increase cell necrosis and decrease proliferation[21-22]. Our data indicated that the exposure to CO₂ decreased the mitochondrial activity of MKN-45 cells, especially in a higher pressure environment (15 mmHg). We noticed this change when it was moved to normal culture environment for 4 h. The percentage of apoptotic cells increased in CO₂ pneumoperitoneum (10 and 15 mmHg group). This phenomenon was also investigated in human ovarian cancer cell lines HO8901, SKVO[22] and other tumor cells[23-24].

Helium has been suggested for alternative use for pneumoperitoneum to prevent CO₂ effects such as local acidosis and systemic hypercapnia[25]. In addition, a beneficial effect of helium versus CO₂ on the growth of rat mammary adenocarcinoma cells was shown in vitro[26]. In our experiments, we observed no obvious difference between helium group and control group, even at 15 mmHg pressure. This proved that the increase of cell apoptotic ratio in CO₂ pneumoperitoneum might not only depend on insufflated pressure.

Kos et al. showed that intracellular and extracellular acidification associated with CO₂ resulted in an attenuation of cytokine release and cell activity in macrophages[27]. Takiguchi et al. believed CO₂ pneumoperitoneum had no effect on cancer cells’ proliferative ratio but had a toxic effect on cancer cells[28].

Our current experiments confirmed that the extracellular pH differed significantly between CO₂ and helium exposure and it decreased very sharply at the insufflated pressure. Wildbrett et al. reported that intracellular and extracellular pH and calcium level were altered with CO₂ pneumoperitoneum[29]. pH and calcium are important regulators of cell functions such as ATP production, cell cycle, intracellular signaling and apoptosis[28,29]. It is likely that all these changes influence the favorability of tumor-cell implantation at the time of laparoscopic surgery.

West et al. excluded hypoxia as a cause of alteration of cell functions by exposing cells to 20% and 80% CO₂[30]. In our experiments, exposure to both 100% CO₂ and 100% helium may cause hypoxia, but the impact on MKN-45 gastric cancer cells was significantly different. Only CO₂ reduced cell activity, which made no hypoxic effects. The direct effects of CO₂ demonstrated by Takiguchi et al. on human colon cancer cells in vitro[31] remain to be confirmed for gastric cancer cells.

CO₂ pneumoperitoneum resulted in severe peritoneal...
acidity, and peritoneal acidosis may play a role in changing tumor cells’ implantation during laparoscopic oncologic surgery. The role of peritoneal microenvironment in tumor-cell growth awaits further studies. More studies in the area could enable us to find the safest approach to laparoscopic oncologic surgery.

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