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RAPID COMMUNICATION

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

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Abstract

AIM: To investigate the effects of carbon dioxide (CO_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_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. Apoptotic ratio was measured by Annexin V-FITC/PI double labelled staining.

RESULTS: The pH of media was acid and recovered to normal after 4 h in the CO_2 group while it was basic in the helium group. There was no difference between CO_2 groups (under 10 mmHg) and control group ($P > 0.05$) in the proliferative viability of the cells. The cultured cells exposed to 15 mmHg CO_2 environment grew more slowly than control group from 4 to 7 d ($P < 0.01$) while there was no difference from 1 to 3 d ($P > 0.05$). The proliferative 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_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_2 insufflation pressure and helium in any pressure. Fifteen mmHg CO_2 insufflation pressure can inhibit the proliferation of the cells and improve apoptosis.

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

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INTRODUCTION

Minimally invasive techniques are increasingly applied in abdominal surgeries[1,2]. In recent years, numerous authors reported an acceptable feasibility of minimally invasive techniques for biopsy and resection of various malignant tumors[3,4]. However, laparoscopic resection for intraperitoneal malignancies remains controversial. One of the reasons is the concern whether carbon dioxide (CO_2) peumoperitoneum can improve cancer cells' growth[5,6]. There is an ongoing debate about the deleterious effects of CO_2 on tumor cell behavior. Some authors showed an increase in cell proliferation and tumor growth[7] and others found beneficial effects of CO_2 exposition *in vitro* and in animal studies[8,9].

It is well known that intracellular and extracellular pH in the peritoneum is affected by CO_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_2 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_2 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℃. The pH of the media was examined using an arterial blood gas analyzer (Radiometer ABL 505, Denmark). After the cells were exposed to CO_2 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 CO_2 and helium group is shown in Tables 1 and 2. When the pressure of CO_2 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).

Table 1 Changes of culture media pH in CO_2 groups (n = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.02	7.18 ± 0.02	7.15 ± 0.01	7.16 ± 0.03	7.20 ± 0.02
0 mmHg	7.13 ± 0.04[b]	7.15 ± 0.03	7.15 ± 0.03	7.19 ± 0.01	7.23 ± 0.04
5 mmHg	7.00 ± 0.05[b]	7.13 ± 0.03	7.22 ± 0.02	7.22 ± 0.02	7.24 ± 0.02
10 mmHg	6.77 ± 0.03[b]	6.95 ± 0.05[b]	7.16 ± 0.03	7.22 ± 0.01	7.21 ± 0.01
15 mmHg	6.18 ± 0.02[b]	6.91 ± 0.02[b]	7.08 ± 0.04	7.20 ± 0.02	7.22 ± 0.01

[b]$P < 0.01$ *vs* control group.

Table 2 Changes of culture media pH in helium groups (n = 4) (mean ± SD)

Groups	Time (h)				
	0	2	4	6	8
Control	7.20 ± 0.01	7.18 ± 0.02	7.15 ± 0.01	7.17 ± 0.03	7.20 ± 0.02
0 mmHg	7.42 ± 0.02[b]	7.23 ± 0.03	7.18 ± 0.01	7.15 ± 0.01	7.16 ± 0.03
5 mmHg	7.53 ± 0.03[b]	7.28 ± 0.02[b]	7.21 ± 0.02[a]	7.19 ± 0.03	7.15 ± 0.01
10 mmHg	7.82 ± 0.02[b]	7.31 ± 0.01[b]	7.23 ± 0.02[b]	7.20 ± 0.05	7.15 ± 0.02
15 mmHg	8.19 ± 0.04[b]	7.96 ± 0.03[b]	7.33 ± 0.05[b]	7.22 ± 0.01	7.19 ± 0.02

[a]$P < 0.05$ *vs* control group, [b]$P < 0.01$ *vs* control group.

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 CO_2 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 CO_2 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 significant difference from the 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

Table 3 Changes of MKN-45 proliferative viability in CO_2 groups (OD, mean ± SD)

Groups	Time (d)						
	1	2	3	4	5	6	7
Control	0.31 ± 0.04	0.41 ± 0.02	0.53 ± 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	1.58 ± 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	0.53 ± 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 ± 0.04[b]	0.80 ± 0.04[b]	1.16 ± 0.08[b]	1.42 ± 0.02[b]

[b]$P < 0.01$ *vs* control group; OD: Optical density.

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

Groups	Time (d)						
	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 apoptosis ratio in CO_2 and helium groups (%, mean ± SD)

Groups	Control	0 mmHg	5 mmHg	10 mmHg	15 mmHg	F
CO_2	0.21 ± 0.02	0.19 ± 0.04	0.29 ± 0.05	9.20 ± 0.44[a]	11.60 ± 0.95[a]	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

[a]$P < 0.05$ *vs* control group; CO_2: 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 CO_2 pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence[5,16,17].

The results of experimental studies on the behavior of tumor cells exposed to CO_2 are not conclusive. Numerous authors confirmed a CO_2 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 CO_2 pneumoperitoneum could increase cell necrosis and decrease proliferation[8,21]. Our data indicated that the exposure to CO_2 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 CO_2 pneumoperitoneum (10 and 15 mmHg group). This phenomenon was also investigated in human ovarian cancer cell lines HO8901, $SKVO_3$[22] and other tumor cells[23,24].

Helium has been suggested for alternative use for pneumoperitoneum to prevent CO_2 effects such as local acidosis and systemic hypercapnia[25]. In addition, a beneficial effect of helium versus CO_2 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 CO_2 pneumoperitoneum might not only depend on insufflated pressure.

Kos *et al* showed that intracellular and extracellular acidification associated with CO_2 resulted in an attenuation of cytokine release and cell activity in macrophages[27]. Takiguchi *et al* believed CO_2 pneumoperitoneum had no effect on cancer cells' proliferative ratio but had a toxic effect on cancer cells[18].

Our current experiments confirmed that the extracellular pH differed significantly between CO_2 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 CO_2 pneumoperitoneum[10]. 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% CO_2[30]. In our experiments, exposition to both 100% CO_2 and 100% helium may cause hypoxia, but the impact on MKN-45 gastric cancer cells was significantly different. Only CO_2 reduced cell activity, which made no hypoxic effects.The direct effects of CO_2 demonstrated by Takiguchi *et al* on human colon cancer cells *in vitro*[18] remain to be confirmed for gastric cancer cells.

CO_2 pneumoperitoneum resulted in severe peritoneal

acidosis, 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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COMMENTS

Background

Laparoscopic surgery in oncologic patients is increasingly adopted as an alternative to conventional surgical procedures, both for diagnosis and resection. However, some experimental and clinical studies have suggested that the CO_2 pneumoperitoneum influences the development of intra-abdominal tumor dissemination and port site metastases. Numerous authors confirmed a CO_2 associated increase of tumor growth and invasiveness of various cell lines derived from colon carcinoma, adenocarcinoma, and other tumors. However, other studies showed beneficial effects of CO_2, such as increased cell necrosis and decreased proliferation.

Research frontiers

The effects of laparoscopic environment on tumor cell biology, including the kind of gas and the pressure of pneumoperitoneum.

Innovations and breakthroughs

The results of experimental studies on the behavior of tumor cells exposed to CO_2 are not conclusive. In this study, the authors elaborately and clearly demonstrate that it is the CO_2 gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

Applications

Laparoscopic resection for intra-abdominal malignancies remains controversial, especially for advanced gastric cancer. One of the reasons is the concern whether CO_2 pneumoperitoneum can improve port-site metastasis, peritoneal dissemination and recurrence. This research on CO_2 pneumoperitoneum could improve the application of CO_2 as the insufflation gas in laparoscopic surgery.

Terminology

Helium insufflation: The act of blowing helium into any body cavity for experimental, diagnostic, or therapeutic purposes. CO_2 pneumoperitoneum: The presence of CO_2 in the peritoneal cavity. It may occur spontaneously or be deliberately introduced as an aid to operate.

Peer review

This is a good study. As far as the *in vitro* effects of gases and pressure on cancer cell growth and apoptosis is concerned, one can find studies reporting exactly contradictory findings. The authors elaborately and clearly demonstrate that it is the CO_2 gas and not the pressure or the hypoxia that inhibits the growth of the cancer cells and increases apoptosis.

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