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Effects of CO_2 pneumoperitoneum on proliferation, apoptosis, and migration of gastrointestinal stromal tumor cells

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Abstract

Background The purpose of the study was to investigate the proliferation and migration capability of human gastrointestinal stromal tumor line GIST-T1 after exposure to different pressures and times of CO_2 pneumoperitoneum.

Methods We established simulated CO_2 pneumoperitoneum environment in vitro and divided the human GIST cell GIST-T1 into open control group, 8 mmHg CO_2 pneumoperitoneum treatment group and 15 mmHg CO_2 pneumoperitoneum treatment group. Each group was divided into two subgroups respectively cultured for 1 h and 3 h. pH value of cell culture, cell growth curve, and cell cycle distribution of each group was measured. By application of scratch healing tests and Transwell chamber experiments, mobility ratio and number of cells through 8 μm membranes were measured to assess the migration ability of cells in each group after intervention.

Results Cell culture pH value of each subgroup in CO_2 group decreased significantly after exposed in CO_2 pneumoperitoneum ($P<0.01$). The proliferation of GIST-T1 cells in 15 mmHg CO_2 group was significantly inhibited early (1–2 days) ($P<0.05$) and the proliferation of GIST-T1 cells in 8 mmHg CO_2 1 h subgroup and 15 mmHg CO_2 1 h subgroup was increased significantly late (4–6 days) ($P<0.05$) after the interventions of CO_2 pneumoperitoneum. The percentage of cells in G0–G1 phase increased, the percentage of S phase cells decreased ($P<0.01$) in 1-h subgroup and 3-h subgroup of 15 mmHg CO_2 group 24 h after exposure to CO_2. The percentage of cells in S phase increased in 1-h subgroup of 8 mmHg CO_2 group and decreased in 3-h subgroup of 15 mmHg CO_2 group 72 h after exposure to CO_2. In the Transwell chamber experiment, the cell number through 8-μm membrane increased significantly ($P<0.01$) in 3-h subgroup of CO_2 group compared to that in 3-h subgroup of control group.

Conclusions The routine pressure and duration of CO_2 pneumoperitoneum used in clinic did not promote the proliferation of gastrointestinal stromal tumors, but had a potential risk of increasing postoperative recurrence and distant metastasis.

Keywords Gastrointestinal stromal tumors · CO_2 pneumoperitoneum · Human gastrointestinal stromal tumor cell GIST-T1 · Proliferation · Migration

Since the first television laparoscopic cholecystectomy was completed by Dr. Lyon Mouret in 1987, laparoscope as the main strategy of the minimally invasive techniques has been 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. The advantages of laparoscopic surgery include less postoperative pain, acceleration of recovery and better cosmetic results [3]. There were a number of randomized clinical trials which showed that laparoscopic operation had similar efficacy to open operation in terms of long-term survival [4]. However, there are some ongoing arguments about the safety of laparoscopy, especially the deleterious effects of CO_2 on tumor

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cell behavior, where some authors have shown an increase in cell proliferation and tumor growth [5, 6], while others found beneficial effects of CO_2 exposition in vitro and in animal studies [7–9].

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract and account for 1–3% gastrointestinal malignancies [10, 11]. Compared to epithelial derived tumors (such as gastric cancer, colorectal cancer, liver cancer, etc.), GISTs have their own characteristics in tissue origin, specific protein expression and molecular biological structure. GISTs occur predominantly in the stomach (50–60%) and small intestine (30–35%) and are thought to originate from interstitial cell of Cajal (ICC) or gastrointestinal mesenchymal stem cells. Recent investigations showed that 80–90% GISTs are mutated in the fibroblast growth factor receptor gene, KIT, 5–10% are mutated in the blood platelet endogenous growth factor receptor, and another 5–10% are mutated in the wild type KIT and PDGFRα gene [12–14]. KIT/PDGFRA activation drives a number of downstream pathways associated with cell proliferation and malignant transformation including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways [15–17]. When the diagnosis of GIST is confirmed or suspected, complete surgical resection must be performed as the definitive treatment. Since GISTs rarely invade and metastasize to lymph node, they are often treated by wedge resection or local resection without requiring large resection margins or extensive lymphadenectomy [18, 19]. Laparoscopic surgery with its attendant benefits lends itself to the resection of GISTs [20]. An increasing number of laparoscopic experiences have been reported to demonstrate the feasibility and safety of resection for gastric GISTs [21–24].

However, questions have arisen over postoperative intraperitoneal or port-site implantation metastasis, which affect the surgical prognosis of GIST. Up to now, the experimental study of the effect of carbon dioxide pneumoperitoneum on tumor cells mainly confined to epithelial malignant tumor cell. To our knowledge, the impact of carbon dioxide on the biological behavior of mesenchymal tumors, such as gastrointestinal stromal tumor, abdominal malignant mesothelioma, has not been investigated. Our study which was in vitro aimed to investigate the proliferation and migration capability of human gastrointestinal stromal tumor line GIST-T1 after exposure to different pressures and duration of CO_2 pneumoperitoneum. This approach may provide the theoretical evidence for exploring the safety of laparoscopic surgery in the treatment of GISTs.

Materials and methods

Cell culture

Human gastrointestinal stromal tumor cell line GIST-T1, originated from the primary tumor of a metastatic primary GIST, was purchased from Shanghai Bogu Biotechnology Company. GIST-T1 cells were cultured in high (4500 mg/L) glucose Dulbecco's Modified Eagle's Medium(DMEM; HyClone, Logan, UT, USA) with 100 g/L fetal bovine serum (Gibco BRL, Gaithersburg, Maryland), 100 IU/mL penicillin G and 100 µg/mL streptomycin sulfate (Gibco BRL). The cells were passaged and maintained in a humidified incubator at 37 °C with 5% CO_2. When the cells had grown to 85% confluence, they were washed in phosphate-buffered saline (PBS), exposed to 0.25% trypsin solution (Gibco BRL), centrifuged at 1000 rpm and resuspended in medium to the desired concentration. Viability was checked by using trypan blue staining and only cells, which viability over than 85%, were used for the experiments.

CO_2 pneumoperitoneum model in vitro

An in vitro CO_2 pneumoperitoneum model was designed for the experimental. We used a cylindrical closed transparent organic glass container which was composed of a tank body and a cover with a sealing device. The cover is provided with two holes, one for the air inlet and the other for the air outlet. A Wolf CO_2 insufflator, used as an automatic pneumoperitoneum machine for laparoscopic operation, was connected to the air inlet hole on the cover by a hose with filtration device. The flow of CO_2 and the pressure of CO_2 in the tank were regulated by the automatic pneumoperitoneum machine. Through a hose with a switch, the air vent hole was connected with the vacuum pump (Fig. 1A, B).

Before the experiment, the pneumoperitoneum tank was cleaned by 75% alcohol, and then was disinfected by UV on the clean bench for 6 h. GIST-T1 cells were inoculated on 96/6-well plates and exposed to the environment of the CO_2 pneumoperitoneum. When the experiment was carried out, the GIST-T1 cells located in the plates were placed inside the pneumoperitoneum tank and the sealing cover was closed. We opened the vent hole which was connected to the vacuum pump and made the tank vacuum. Close the venthole and then high purity medical CO_2 gas was infused into the pneumoperitoneum box through inlet hole by the automatic pneumoperitoneum machine. CO_2 environment was adjusted to 8 or 15 mmHg for periods of 1 or 3 h according to experimental protocols (Fig. 1C, D).

Fig. 1 CO_2 Pneumoperitoneum model in vitro (**A** overall connection of automatic pneumoperitoneum machine and cylindrical closed transparent organic glass container; **B** closed transparent organic glass container. The blue interface connected to the automatic pneumoperitoneum machine was for the air inlet and the white interface connected to a vacuum pump or closed was for air outlet; **C** When the experiment was carried out, the GIST-T1 cells located in the plates were placed inside the pneumoperitoneum tank and the sealing cover was closed; **D** during the experiment, high purity medical CO_2 gas was infused into the pneumoperitoneum tank through inlet hole by the automatic pneumoperitoneum machine). (Color figure online)

Experiment design

To investigate the effect of pressure or duration in CO_2 environment on GIST-T1 cells, the cells were divided into three groups as follows: (1) Control group: the 96/6-well plates inoculated with GIST-T1 cells were place on the clean bench under the normal atmospheric pressure and the temperature of 26 °C, which simulated the actual open operation environment; (2) High-pressure CO_2 pneumoperitoneum group: the 96/6-well plates inoculated with GIST-T1 cells were placed in the pneumoperitoneum tank and exposed to the environment of CO_2 pneumoperitoneum with 15 mmHg pressure at a normal temperature of 26 °C; (3) Low-pressure CO_2 pneumoperitoneum group: the 96/6-well plates inoculated with GIST-T1 cells were placed in the pneumoperitoneum tank, maintaining the pressure at 8 mmHg and a normal temperature of 26 °C. Each group was divided into two subgroups, which were treated for 1 h and 3 h respectively in the same treatment environment. After the intervention, the GIST-T1 cells were cultured in a humidified incubator at 37 °C with 5% CO_2 and the medium was replaced after 24 h. The medium pH values in each group were measured by electronic pH meter (OHAUS, USA) before and after disposal at 0, 1, 2, 3, 4, 5 h. Our study was a cytological experiment in vitro and it did not involve human or animal subjects, so we did not apply the ethical approval in our institution.

In vitro cell proliferation assay

Cell proliferation was assessed by cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay according to the manufacturer's instruction. GIST-T1 cells in the logarithmic growth phase were concentrated to 2.5×10^4/mL. The cells were seeded on 96-well plates and 100 μL of cells was inoculated into one hole (2500 cells per hole), which allowed cells to grow for 7 days. The plates were placed in an incubator for 24 h and then separated into their groups and treated according to the experimental protocols. The CCK-8 solution was added in each well before and after disposal at 0, 1, 2, 3, 4, 5, 6, 7 days. Then the cells were incubated at 37 °C in a 5% CO_2 incubator for 1 h. The absorbency was measured at a test wavelength of 450 nm and a reference wavelength of 630 nm using a microplate reader (Bio-Rad). Eight duplicate

wells were used for each experimental subgroup, and the experiment was repeated three times.

Cell cycle analysis

GIST-T1 cells (10×10^4 cells/well) were seeded in a six-well microplate. After 24 h of stabilization, the plates were separated into their groups and treated according to the experimental protocols. At 24, 72 h after the treatment, cells in each group were harvested, washed once with PBS and fixed with 70% cold ethanol overnight. Fixed cells, washed once with PBS, were resuspended in propidium iodide (PI) buffer (100 μg/mL PI, 50 μg/mL RNAse A in PBS) and incubated for 15 min on ice in the dark. Cell cycle distribution was detected using a flow cytometer (FACS Calibur SE; BD Biosciences, San Jose, CA, USA). Data were analyzed using Multicycle DNA cell cycle analysis software and represented as percent cells in G0/G1, S, and G2/M phase. For each sample, 10,000 events were analyzed in three independent experiments for each subgroup at each timepoint.

Wound-healing assay

Migration status of GIST-T1 cells was detected by the wound-healing assay. After GIST-T1 cells grown in six-well plates had reached 90% confluence, the plates were separated into their groups and treated according to the experimental protocols. After the treatment, a scratch was made with a standard 200-μL pipette tip followed by extensive washing with serum-free medium to remove cell debris. Then fresh medium was added and cells were cultured for another 48 h. The width of the scratch gap was observed using an inverted microscope (Carl Zeiss AG, Germany) with 3 randomly selected fields in the wounded region at 0 h, 24 h, and 48 h after the artificial wound creation. The percentage of wound width was calculated as follows: the wound width at 0, 24, or 48 h/the original wound width measured at 0 h. Three replicate wells were performed for each experiment in each subgroup.

Transwell migration assay

Cell migration was assessed using a 24-well Transwell plate with 8-μm polycarbonate sterile membranes (Corning Inc., Corning, NY, USA).GIST-T1 cells (10×10^4 cells/well) were seeded in a six-well microplate. After 24 h of stabilization, the plates were separated into their groups and treated according to the experimental protocols. After disposal, cells in each group were harvested, washed once with PBS. GIST-T1 cells of each subgroup (2×10^5 cells/mL) suspended in 200 μL serum free high glucose DMEM were planted in the top chamber of the Transwell, and 500 μL high glucose DMEM with 10% FBS was added to the lower chambers. The cells were allowed to migrate for 24 h. At the end of the culture period, the cells on the upper surface were detached with a cotton swab. The filters were fixed in 4% formaldehyde for 10 min, and cells in the lower filter were stained with 0.1% crystal violet for 15 min and counted. The numbers of the migrating cells in each well were counted in 6 random microscopic fields per filter at 200× magnification. The experiments were performed in triplicate independently for each subgroup.

Statistical analysis

The experimental data were analyzed by SPSS17.0 and shown as the mean ± standard deviation. Intergroup differences of cell proliferation assay, cell cycle analysis, and Transwell migration assay in the experiment were detected by analysis of variance (ANOVA). When ANOVA showed a statistically significant difference, a group-by-group comparison was performed using Least-Significant Difference (LSD) test. The result of wound-healing assay was compared using analysis of variance for repeated measurement data. P values less than 0.05 were considered significant.

Results

Influence of CO_2 pneumoperitoneum in pH of media

There was an acidifying effect of CO_2 pneumoperitoneum on the cell culture media. As shown in Fig. 2, the pH values of media tended to decrease with the increase in CO_2 pressure and exposure time. Immediately after treatment, the pH values of media from CO_2 groups decreased significantly (compared to control group, $P<0.01$), among which changes in 3 h subgroup of 15 mmHg CO_2 group was most obvious, down to 6.14. The pH values increased up to the basal level of the control group gradually when the cells were moved to normal culture environment. Figure 2 shows that the lower the pressure and the shorter the exposure time, the faster the recovery of pH value. 3 h after treatment, the pH of the media in 1 h subgroup of 8 mmHg CO_2 group increased up to about the basal level of the control group ($P>0.05$). At 5-h post-exposure, there was no significant difference in the pH of the media among the treatment groups and the control group ($P>0.05$).

Effect of CO_2 pneumoperitoneum on cell viability using the CCK-8 method

Compared to the control group, the proliferative viability of GIST-T1 cells in the 1-h subgroup was significantly increased from d4 to d6 after it was exposed to 8 mmHg CO_2 pneumoperitoneum ($P<0.05$). A significant decrease

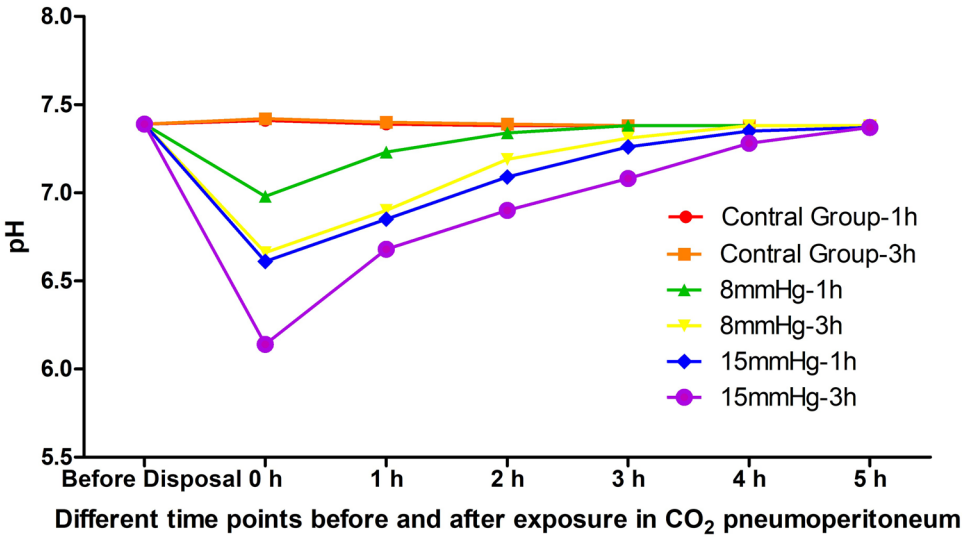

Fig. 2 Influence of CO_2 pneumoperitoneum in pH of media

of cell activity was determined in 1-h subgroup at d1 and in 3-h subgroup from d1 to d2 after exposed to 15 mmHg CO_2 pneumoperitoneum at ($P < 0.05$). 5 days after exposed to 15 mmHg CO_2 pneumoperitoneum, the cell viability in 1-h subgroup was significantly higher than that in the control group ($P < 0.05$) (Fig. 3A).

Compared to shorter exposure time (1 h) in CO_2 pneumoperitoneum with the same pressure, longer time exposure (3 h) could decrease proliferative capabilities of GIST-T1 cells. It was observed from d4 to d6 after disposed with 8 mmHg CO_2 pneumoperitoneum and at day 1, 2, 4 after exposed to 15 mmHg CO_2 pneumoperitoneum ($P < 0.05$) (Fig. 3D, E). Compared to higher pressure (15 mmHg) CO_2 pneumoperitoneum with the same exposure time, lower pressure (8 mmHg) CO_2 pneumoperitoneum could increase proliferative capabilities of GIST-T1 cells. It was observed in 3-h subgroup from d1 to d2 after exposed to CO_2 pneumoperitoneum and in 1 h subgroup at d1, d4, d6 after disposed with CO_2 pneumoperitoneum ($P < 0.01$) (Fig. 3B, C).

Effects of CO_2 pneumoperitoneum on the cell cycle distribution of GIST-T1 cells

Flow cytometry was performed to investigate the alterations in cell cycle distribution at two time point (24 h, 72 h) after treatment of CO_2 pneumoperitoneum (Fig. 4). We found that there was no significant difference in the cell cycle distribution between control group and 8 mmHg CO_2 pneumoperitoneum group at 24 h after exposure ($P > 0.05$). Compared to the control group, the number of cells in G0/G1 phase was significantly higher in 15 mmHg CO_2 pneumoperitoneum group at 24 h after exposure ($P < 0.01$). The increase in G0/G1 phase was accompanied by a decrease in the frequency of cells in the S phase ($P < 0.01$). Cells exposed to 8 mmHg CO_2 pneumoperitoneum for 1 h showed a significant decrease in G0/G1 phase and a significant increase in S phase at 72 h after exposure ($P < 0.01$). At 72 h after exposure to 15 mmHg CO_2 pneumoperitoneum for 1 h, the cell population showed a similar pattern of behavior with a significant decrease in G0/G1 phase and a significant increase in S phase ($P < 0.05$). The exposure of the cells to 15 mmHg CO_2 pneumoperitoneum for 3 h caused a significant increase of cells in the G2/M phase and a significant decrease in S phase at 72 h after disposure ($P < 0.05$) (Fig. 4).

Compared to shorter exposure time (1 h) in CO_2 pneumoperitoneum with the same pressure, longer time exposure (3 h) causes a significant increase of cells in the G0/G1, G2/M phase and a significant decrease in the S phase ($P < 0.05$). Compared to higher pressure (15 mmHg) CO_2 pneumoperitoneum with the same exposure time, lower pressure (8 mmHg) CO_2 pneumoperitoneum could cause cells to arrest in the S phase of the cell cycle. It was observed in 1-h subgroup at 24 h after exposed to CO_2 pneumoperitoneum and in 3-h subgroup at both the time points (24 h, 72 h) after disposed with CO_2 pneumoperitoneum ($P < 0.05$) (Fig. 4).

Effects of CO_2 pneumoperitoneum on GIST-T1 cell migration ability

In order to determine the effect on the migration ability of GIST-T1 cells, a wound-healing assay and a two-chamber Transwell assay were performed. As shown in Fig. 5, no detectable differences in the percentage of wound width were observed among the control group, 8 mmHg CO_2 pneumoperitoneum group and 15 mmHg CO_2 pneumoperitoneum group ($P > 0.05$). As suggested by the transwell migration assay, there were significantly more migrated cells in the 3 h subgroup of 8 mmHg CO_2 pneumoperitoneum group and

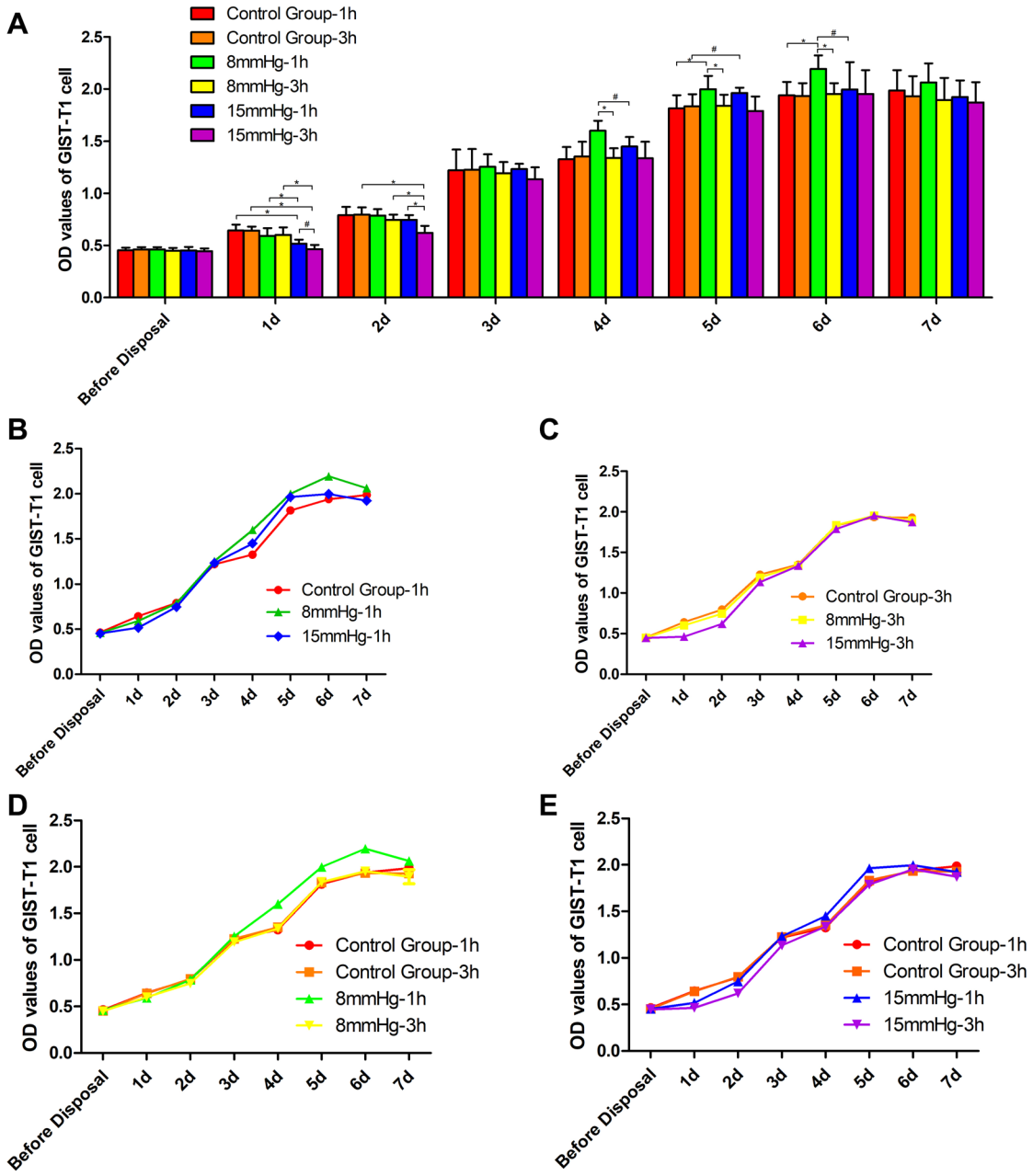

Fig. 3 Effect of CO_2 pneumoperitoneum on cell viability using the CCK-8 method (**A** effect of CO_2 pneumoperitoneum on GIST-T1 proliferation activity with different pressures and different intervention times. #$P<0.05$, *$P<0.01$; **B** the growth curves of GIST-T1 after 1-h exposure in CO_2 pneumoperitoneum with different pressures; **C** the growth curves of GIST-T1 after 3-h exposure in CO_2 pneumoperitoneum with different pressures; **D** the growth curves of GIST-T1 after exposure in 8 mmHg CO_2 pneumoperitoneum with different intervention times; **E** the growth curves of GIST-T1 after exposure in 15 mmHg CO_2 pneumoperitoneum with different intervention times.)

15 mmHg CO_2 pneumoperitoneum group than in the control group ($P<0.01$) (Fig. 6).

Compared to shorter exposure time (1 h) in CO_2 pneumoperitoneum with the same pressure, GIST-T1 cells with long exposure time (3 h) were significantly promoted to migrate into the lower chamber of the Transwell assay ($P<0.05$). Compared to low pressure (8 mmHg) CO_2 pneumoperitoneum with the same exposure time, the numbers of GIST-T1 cells crossing the chambers increased significantly after exposed in high pressure (15 mmHg) CO_2 pneumoperitoneum for 3 h ($P<0.01$) (Fig. 6).

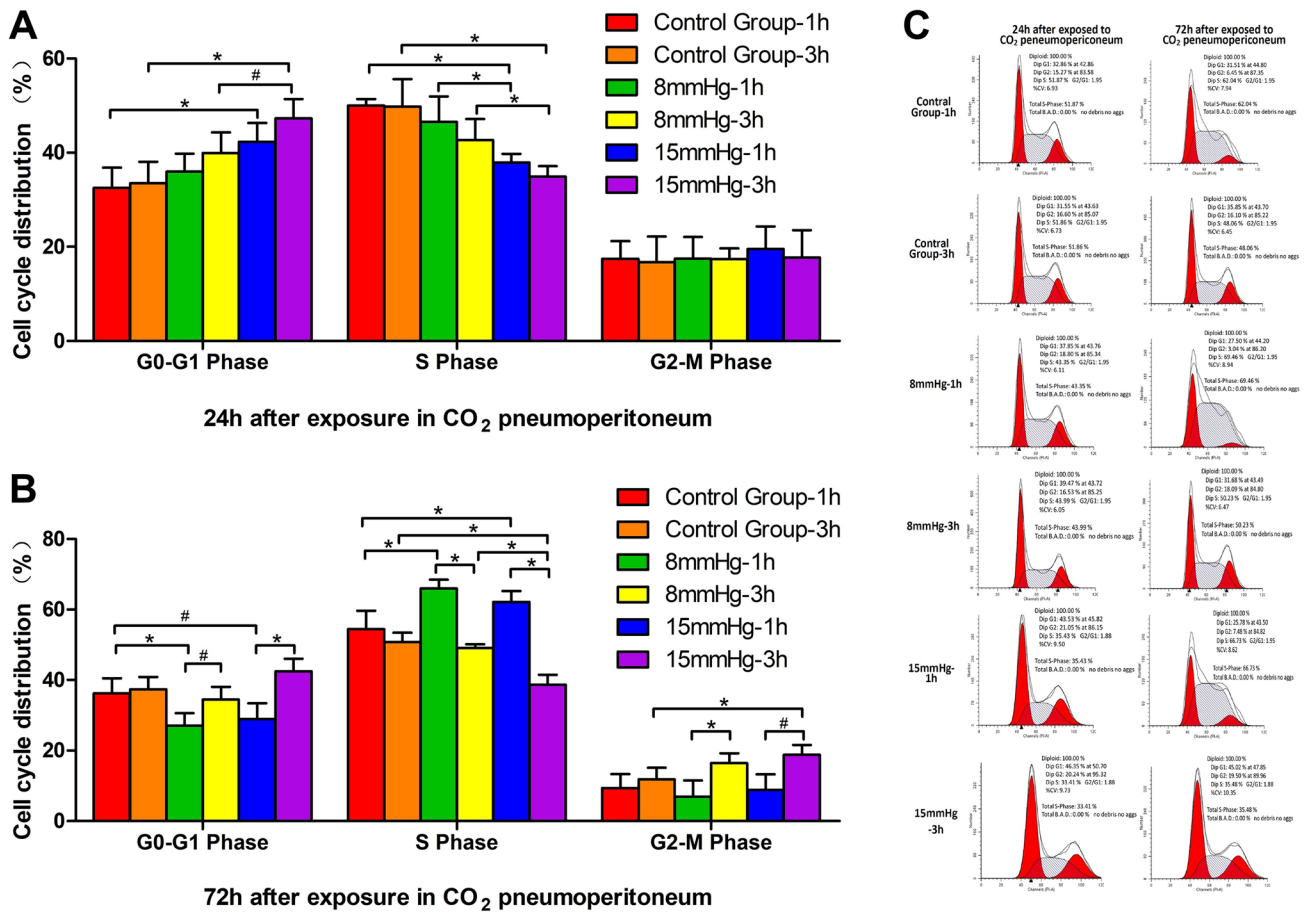

Fig. 4 Effect of CO_2 pneumoperitoneum on the cycle distribution of GIST-T1 cells with different pressures and different intervention times. (**A** Effect of CO_2 pneumoperitoneum on the cycle distribution of GIST-T1 cells at 24 h after exposure. #$P<0.05$, *$P<0.01$; **B** effect of CO_2 pneumoperitoneum on the cycle distribution of GIST-T1 cells at 72 h after exposure. #$P<0.05$, *$P<0.01$; **C** the result of flow cytometer to detect the cell cycle distribution of GIST-T1 cells with different pressures and different intervention times.)

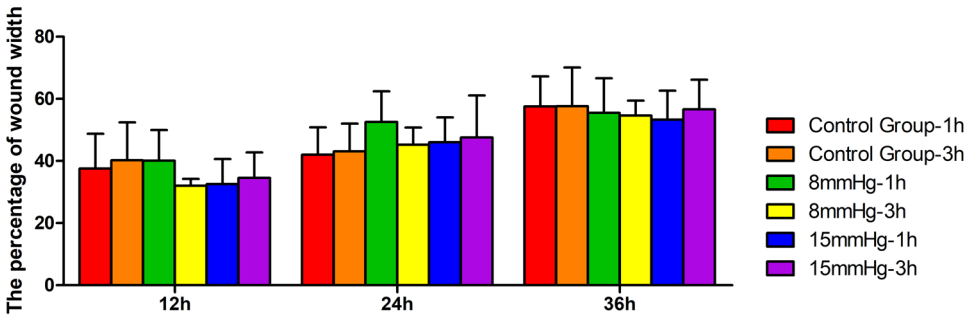

Fig. 5 Comparison of cell migration rates of GIST-T1 cells in a wound-healing assay at different time points after exposed in CO_2 pneumoperitoneum with different pressures and durations

Discussion

Since Dr. Lyon Mouret in France completed the first television laparoscopic cholecystectomy in 1987, laparoscope has been widely used in abdominal surgery. It becomes the main strategy of the minimally invasive techniques in the field of general surgery, obstetrics and gynecology, urology surgery with the improving of laparoscopic instruments and surgical techniques [25–27]. In recent years, laparoscopic surgery is no longer limited to the surgical treatment of various benign diseases [28, 29]. Laparoscopy has been used in the management of gastric cancer and colorectal cancer for the past 10 years because of its advantages including less postoperative pain, shorter recovery period, and better cosmetic effects [30, 31]. However, laparoscopic resection for intraperitoneal malignancies has remained controversial, since Dobronte first

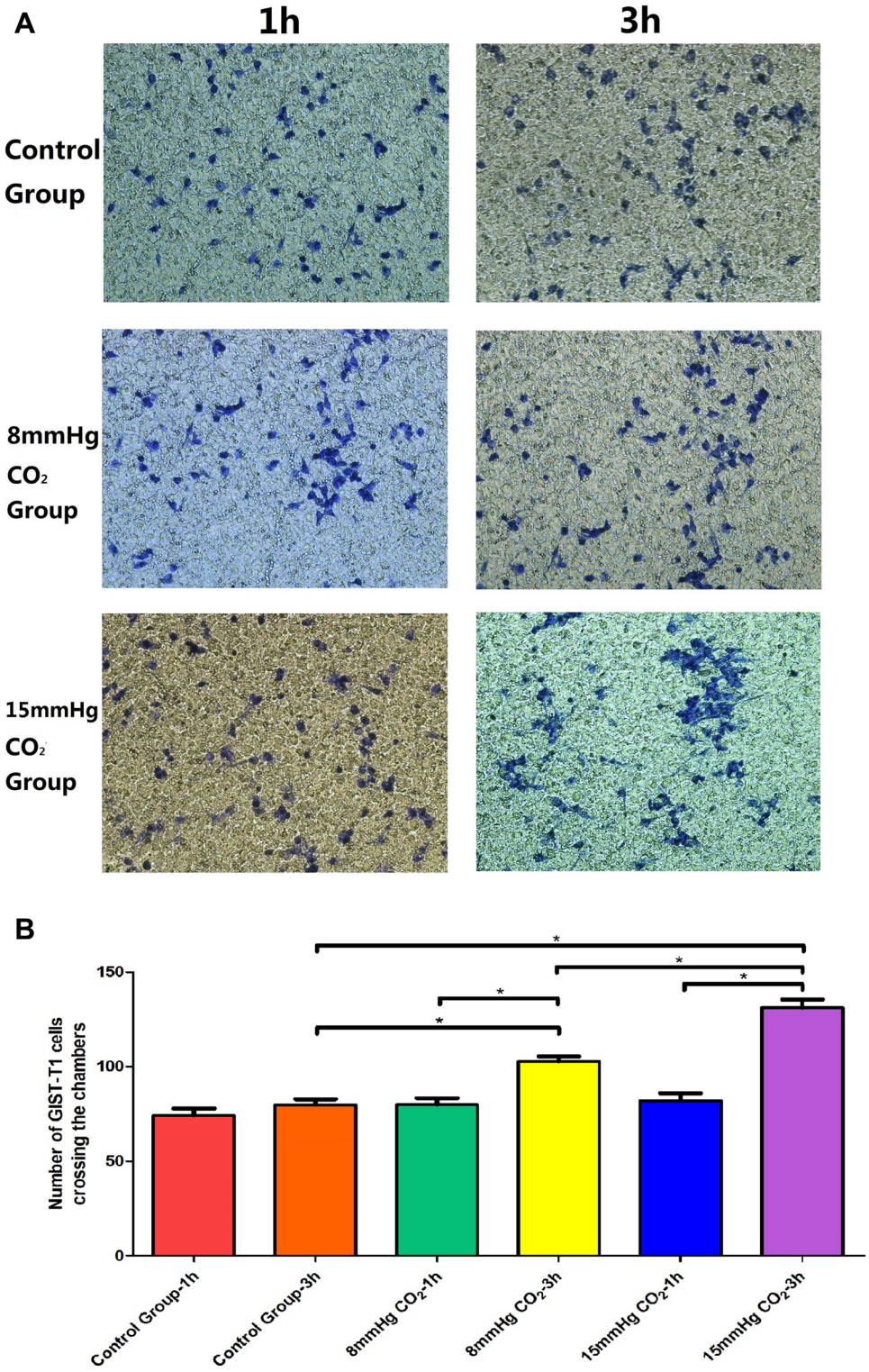

Fig. 6 Effect of CO_2 pneumoperitoneum on the migration ability of GIST-T1 cells suggested by a two-chamber Transwell assay. (**A** GIST-T1 cells in different groups migrated into the lower chamber of the Transwell assay (Crystal violet staining, ×100). **B** Comparison of the migration ability of GIST-T1 cells with different pressures and durations in Transwell assay. $*P < 0.001$)

reported tract recurrence and port site metastasis following laparoscopic resection of malignant tumors in 1978 [32]. The safety of laparoscopy, especially whether carbon dioxide (CO_2) pneumoperitoneum can promote the growth and metastasis of tumor cells, has been a matter of debate [33]. But the results of numerous experimental studies were not conclusive. Some authors showed that CO_2 insufflation during laparoscopy can increase tumor growth and induce metastases at the punctures and dissemination through the abdominal cavity [34]; others found no differences in

terms of recurrence and metastases of malignancy between laparotomy and laparoscopy [9], even beneficial effects of CO_2 exposure in vitro and in animal studies [7].

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract [35]. Because of the low frequency of lymph node involvement and spreading beyond the neighboring tissue, the treatment of choice for localized GIST is complete surgical resection with clear margins [19, 21, 36]. The recommended simple surgical approach has made laparoscopic resection widely used in the treatment for GIST [21–24]. Surgical resection in patients with primary GIST is associated with a 5-year survival rate of 48–70% [18, 37–39]. Unfortunately, despite a histopathologically complete tumor resection, up to 50% of GIST will recur at a median of 24 months [18]. Since most recurrences occur along the peritoneal and serosal surfaces, the critical question whether the correlative factors of the laparoscopic techniques, especial CO_2 pneumoperitoneum, could affect the biological behavior of GIST has been concerned. However, there are limited data on the oncological impact of CO_2 insufflation in GISTs.

CO_2 is currently the insufflation gas of choice for laparoscopy. It fulfills most requirements for an ideal insufflation gas, which is colorless, noninflammable and rapidly excreted from the circulation. Laparoscopic operation with CO_2 is known to cause a preoperative increase in arterial blood pCO_2 and acidosis [40, 41], which is typically balanced with increased mechanical ventilation. Recent data show that intracellular and extracellular pH in the peritoneum and subcutaneous tissue is affected by CO_2 insufflation [42]. The acidification was most prominent in the peritoneal fluid, leading to the conclusion that there is a considerable change in the local peritoneal acid–base balance during laparoscopic operation.

To simulate the environment produced during laparoscopic operation, we designed an in vitro pneumoperitoneum device which can simulate different stable pressures by connecting a CO_2 gas-sealed tank with the automatic CO_2 pneumoperitoneum machine. By detecting pH value of cell culture medium after exposure to CO_2 pneumoperitoneum with different pressures and different intervention times, we found that the increasing solubility of CO_2 in high pressure CO_2 pneumoperitoneum led to the carbonic acid formation in medium increasing and pH decreasing. After the intervention, the CO_2 in the culture fluid was released rapidly in normal culture environment (37 °C, 5%CO_2) and the concentration of carbonic acid in the culture medium was decreased. The pH values increased up to the basal level of the control group gradually. Therefore, in our experiment, the effect of CO_2 pneumoperitoneum environment on GIST-T1 cells was a transient procedure, which simulated accurately the internal environment change caused by CO_2 pneumoperitoneum in laparoscopic operation for GISTs.

The proliferative ability of tumor cells is the main manifestation of malignant biological behavior. Proliferation ability of gastrointestinal stromal tumor (GIST) which is a tumor with potential malignant biological behavior is an important index to assess its potential malignancy. In this study, we assessed the effect of pneumoperitoneum on the proliferation ability of GIST-T1 cells by using CCK-8 method for the determination of cell growth curve and flow cytometry for the changes of cell cycle. Our result indicated that high pressure and longer time exposure in CO_2 pneumoperitoneum inhibited the growth of human GIST-T1 cells in the early period after intervention and lower pressure and shorter time CO_2 pneumoperitoneum promoted proliferation of human GIST-T1 cells in the later period after intervention.

The changes of physical and chemical factors in the growth environment of tumor cells may be the main cause of the proliferation change of tumor cells under the intervention of CO_2 pneumoperitoneum. The current study found that the inhibition of growth and metabolism of tumor cells in early intervention of CO_2 pneumoperitoneum may be due to the environmental changes. It was previously shown that the release of cytokines and free oxygen radicals, as well as the mitochondrial activity of macrophages and polymorphonuclear cells, is downregulated by CO_2 associated pH-decreases and by direct effects of CO_2 [43, 44]. However, some 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 [34, 45–47]. Some researchers believe that intracellular acidosis occurring during CO_2 insufflation may inhibit this enzyme system. Thus, impaired neutrophil migration and inhibition of the respiratory burst may create conditions favorable for adhesion and growth of liberated tumor cells [48]. Molinas found that CO_2 pneumoperitoneum caused severe hypoxia in tumor cells. Hypoxia inducible factor (HIF) expression and increase in phosphorylation promotes HIF-1 alpha subunit, HIF-1 beta dissociation and combination with P53, which resulted in inhibition of cell apoptosis [49]. Zhu et al. reported that the expression of BCL-2, PCNA, and VEGF increased in tumor cells cultured under anoxic condition. The results suggested that the anti-apoptosis ability, proliferation and angiogenesis of tumor cells in anoxic condition were markedly increased [50].

The ability of migration and movement in tumor cells is one of the causes for malignant tumors to develop, adhere, invade, and metastasis. Studies have shown that the migration speed of tumor cells was positively correlated with the ability of migration and movement in tumor cells. The cells with high invasiveness often had active cell motility. In recent years, a concern was raised by endoscopic surgeons that laparoscopic surgery in the treatment of abdominal malignant tumor might increase the risk of postoperative peritoneal dissemination and metastasis of puncture point

(port site metastasis, PSM) [51–53]. Döbrönte first reported the metastasis of incision or puncture point after laparoscopic resection of malignant tumor [32]. Hao et al. found high pressure (15 mmHg) pneumoperitoneum can inhibit the migration ability of gastric cancer cells in vitro and assumed that the inhibition may be caused by the disruption of the cytoskeleton [54]. It was also found that the migration of lung cancer H128 cells under serious hypoxia was significantly lower than the control group and the deterioration of migration ability was closely related to decreased expression of E-cadherin and integrin beta1 [55].

In this study, we established an artificial CO_2 pneumoperitoneum model in vitro and determined the effects of CO_2 pneumoperitoneum on GIST-T1 cell migration using the method of wound-healing test and Transwell chamber experiment. The wound-healing test showed that the migration rate of GIST-T1 cells in each group had no significant difference at each time point after intervention. However, in the Transwell chamber experiment, the numbers of GIST-T1 cells crossing the chambers increased significantly after exposed in CO_2 pneumoperitoneum for 3 h ($P < 0.01$). Further analysis (comparing the influence of different exposure times with same CO_2 pneumoperitoneum pressure and different CO_2 pneumoperitoneum pressures with same exposure duration on the number of GIST-T1 cells moving through 8-μm membrane in Transwell chamber) indicated that exposure to high pressure CO_2 pneumoperitoneum with long time can obviously promote the migration ability of GIST-T1 cells and increase the number of GIST-T1 cells moving through 8-μm membrane.

With the same experiment material and intervention conditions, wound-healing test and Transwell chamber experiment obtained inconsistent results, which may be related to the migration mode of human gastrointestinal stromal tumor cells in vitro. In the wound-healing test, the migration of cells was mainly carried out in two-dimensional space and the movement of cell migration was mainly guided by cell processes. However, the migration of tumor cells was in the main form of amoeba migration. In the pattern of amoeba migration, the tumor cells were acted by chemotaxis and completed invasion by the matrix adhesion and self-extrusion deformation through the matrix gap [56]. It was difficult for GIST-T1 cells to simulate amoeba migration in the wound-healing test. In Transwell chamber experiment, the holes on the filter plate limited the cells in the above chamber through into the lower chamber. Tumor cells can simulate chemotactic migration in the 3D structure of Transwell chamber experiment [57, 58]. Hence, we believed that exposure in CO_2 pneumoperitoneum with long time (3 h) could obviously promote the migration ability of GIST-T1 cells.

There were several limitations associated with our study. In vitro, human gastrointestinal stromal tumor cells, GIST-T1, adhered to the surface of plastic dishes in a monolayer manner. Under the condition of CO_2 pneumoperitoneum, CO_2 only acted on the tumor cells, but had no significant effect on the supporting tissues attached by the tumor cells. In vivo, CO_2 pneumoperitoneum not only affects the tumor cells, but also affects the local microenvironment, especially the matrix tissue around the tumor. The interaction between CO_2 pneumoperitoneum, tumor cells and surrounding matrix tissue could not be simulated by our experiments in vitro. Cells cultured in vitro grew in cell culture medium. As a weak buffer, the medium had limited resistance to acidosis caused by CO_2 pneumoperitoneum. The change of local environment caused by simulated CO_2 pneumoperitoneum was rigid, i.e., pH value of culture medium decreased obviously during CO_2 pneumoperitoneum intervention and recovered in a short time after culture medium was moved to normal culture environment. However, the buffering mechanism of humoral regulation in vivo was more complex than that in the isolated system. Our CO_2 pneumoperitoneum model in vitro could not completely simulate the effect of buffering mechanism on tumor in vivo. In the future, we will establish an animal model of gastrointestinal stromal tumors and observe the growth, proliferation and metastasis of gastrointestinal stromal tumors after CO_2 pneumoperitoneum intervention in vivo. It will provide more reliable experimental evidence for the safety of laparoscopic surgery in treating gastrointestinal stromal tumors.

Conclusion

Simulated low pressure CO_2 pneumoperitoneum (8 mmHg) with short time could promote the proliferation of GIST-T1 cells cultured in vitro. Long time high pressure CO_2 pneumoperitoneum (15 mmHg) may have a transient inhibition of GIST-T1 cells cultured in vitro proliferation. Exposure in simulated CO_2 pneumoperitoneum with long time (3H) could promote the migration ability of GIST-T1 cells cultured in vitro. The routine pressure and duration of CO_2 pneumoperitoneum used in clinic did not promote the proliferation of gastrointestinal stromal tumors but had a potential risk of increasing postoperative recurrence and distant metastasis.

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Compliance with ethical standards

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