Hyperthermic carbon dioxide pneumoperitoneum reinforces the inhibition of 5-FU on the proliferation and invasion of colon cancer

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Abstract. The effect of hyperthermic carbon dioxide (CO_2) pneumoperitoneum in combination with 5-fluorouracil (5-FU) on the proliferation and invasion of colon cancer was explored. Colon cancer cell line SW-480 was sealed into the urine collection bag to simulate pneumoperitoneum with 100% CO_2 under a pressure of 12 mmHg. The cells were divided into group A, CO_2 at 37˚C; group B, CO_2 at 43˚C; group C, 5-FU; group D, CO_2 at 37˚C+5-FU; group E, CO_2 at 43˚C+5-FU; and control groups under normal culture conditions. The cell proliferation was assessed by CCK-8 test; the cell apoptosis was tested by FACS analysis; the cell invasion was examined by Transwell assay; the expression of HSP-70, caspase-3, HIF-1α and MMP-9 proteins and genes were detected by western blot analysis and RT-PCR. The SW-480 cells were injected into nude mouse cecum subserosal to establish a colon cancer model. We applied 43˚C CO_2 pneumoperitoneum or 5-FU intraperitoneal chemotherapy to intervene, detected the transplantation tumor growth and metastasis. The cell proliferation was inhibited in groups B, C, D and E, apoptosis was induced in groups B, C, D and E, the Transwell cell number decreased in groups B, C, D and E, the transplantation tumor weight and metastasis rate were inhibited in groups B, C, D and E, but all not in group A. The most significant change was observed in group E. Hyperthermic CO_2 pneumoperitoneum was able to reinforce the inhibition of 5-FU on proliferation and invasion of colon cancer.

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Introduction

Laparoscopy has been widely used in colorectal surgery, and CO_2 is commonly used to create laparoscopic pneumoperitoneum. However, controversies exist regarding the effect of CO_2 pneumoperitoneum on tumor proliferation and metastasis. It is believed that CO_2 pneumoperitoneum could potentially promote colon cancer cell proliferation or metastasis under certain conditions (1-3). However, the intraperitoneal hyperthermic chemoperfusion (IHCP) was demonstrated to eradicate free tumor cells and micrometastases, preventing the peritoneal dissemination of tumors, and is commonly used as adjuvant therapy for open surgeries of gastric, colon and ovarian cancers (4). However, the questions that remain open are how to reduce the adverse influence of CO_2 pneumoperitoneum on the therapeutic effect of colon cancer surgery, and how to utilize IHCP as the combined therapy. It was speculated that the therapeutic effect may be improved by combined therapy of hyperthermic CO_2 pneumoperitoneum and intraperitoneal 5-fluorouracil (5-FU) chemotherapy. In the present study, we investigated the combined effect of hyperthermic CO_2 pneumoperitoneum and 5-FU on the proliferation and invasion of colon cancer *in vitro* and *in vivo*.

Material and methods

Cell culture and nude mice. Colon cancer cell line SW-480 was procured from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured with L-15 medium containing 10% calf serum in an incubator at 37˚C supplemented with 5% CO_2, 20% O_2 and 75% N_2. The culture medium was changed every other day, and the cells at logarithmic growth phase were used for experiments. Nude mice were Balb/C, male, age, 4-6 weeks; weight, 18-20 g, total 72, purchased from East China Normal University Minhang Laboratory Animal Center, raised under the condition of SPF.

Equipment and machine. L-15 culture medium was obtained from Gibco (Thermo Fisher Scientific, Waltham, MA, USA), calf serum was from Hangzhou Tianhang Biological Technology Co., Ltd. (Hangzhou, China) and 5-FU was from

Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). The following reagents or kits were used: cell counting kit-8 (CCK-8) cytotoxicity analysis kit, Annexin V-FITC apoptosis detection kit (both from Dojindo Laboratories, Kumamoto, Japan), KGI cell DNA content detection kit and Transwell detection kit (Corning, Inc., Corning, NY, USA), TRIzol reagent (Invitrogen, Carlsbad, CA, USA), RNA extraction kit-RNAiso Plus (Takara, Shiga, Japan), First Strand cDNA synthesis kit (Thermo Fisher Scientific), and Maxima SYBR-Green/ROX qPCR master mix (Thermo Fisher Scientific). The first antibodies were procured from Abcam (Cambridge, MA, USA), including mouse monoclonal antibody against heat shock protein-70 (HSP-70) or hypoxia-inducible factor-1α (HIF-1α), and rabbit monoclonal antibody against mitochondrial membrane potential-9 (MMP-9) or caspase-3. Polymerase chain reaction (PCR) primers were designed and synthesized by Sangon Biotech (Shanghai, China). The equipment included the enzyme-linked immunosorbent assay reader (Thermo Fisher Scientific), flow cytometry (BD FACSCalibur; BD Biosciences, Flanklin Lakes, NJ, USA), western blot electrophoresis (Bio-Rad, Hercules, CA, USA), and fluorescent-PCR machine (Applied Biosystems, Foster City, CA, USA).

Cell treatment and experimental grouping. The disposable 2-L urine collection bag was used to simulate the pneumoperitoneum. A small cut was made on the lateral part of the urine collection bag through which a balanced plate was placed inside and the petri dish or a 96-well plate was attached. Then, the cut was sealed using a sealer. One port of the urine collection bag was connected to 100% CO_2 gas, and the other port was connected to a pressure meter to monitor the pressure of CO_2 at 12 mmHg. The temperature was set at 43 and 37˚C using a cell culture incubator for 2 h. 5-FU was used at a concentration of 30 μg/ml [IC_{50} (5)]. The cells were grouped as follows: control group (ctrl), cells treated with only CO_2 pneumoperitoneum at 37˚C (group A), cells treated with hyperthermic CO_2 pneumoperitoneum at 43˚C (group B), cells treated with 5-FU only (group C), cells treated with CO_2 pneumoperitoneum at 37˚C and 5-FU (group D), and cells treated with hyperthermic CO_2 pneumoperitoneum at 43˚C and 5-FU (group E). The cells were placed back into the normal incubator.

In vivo tumor establishment and grouping. The *in vivo* tumor growth and metastasis assay was approved by the Ethics Committee of the Fifth People's Hospital of Shanghai. The SW-480 single cell suspension was injected into cecum subserosal of Balb/c nude mice to establish *in situ* colon cancer nude mouse model according to the experimental methods of Zheng *et al* (6). We used an in-house device for many mice simultaneously to warm in the CO_2 pneumoperitoneum research experiment (Chinese patent no. ZL201520774334.4) (Fig. 1), we established different pneumoperitoneum intervention. The model mice were grouped as follows: control group (ctrl), CO_2 pneumoperitoneum at 37˚C (group A), hyperthermic CO_2 pneumoperitoneum at 43˚C (group B), 5-FU (group C), CO_2 pneumoperitoneum at 37˚C and 5-FU (group D), and hyperthermic CO_2 pneumoperitoneum at 43˚C and 5-FU (group E). Each group had 12 mice.

Proliferation and morphology of cells. The cells were seeded onto the 6-well plate at a density of $5x10^5$/well in 2 ml. The cells from each group were observed under the microscope and photographed at 12, 24, 36, 48, 60 and 72 h, respectively, after treatment. The experiment was repeated three times.

Cell proliferation inhibition detection by CCK-8 test. The cells ($1x10^4$/well) at logarithmic growth phase were seeded onto the 96-well plate and cultured for 24 h. The cells of each well were treated separately and continuously cultured for 12, 24, 36, 48, 60 and 72 h, respectively. CCK-8 (10 μl) was added to each well for 4 h, avoiding light. The optical density value at 450 nm was measured to determine the number of viable cells. The triplicate wells were used to calculate the average, and the cell proliferation inhibition was calculated to plot the curve of proliferation inhibition.

Cell apoptosis detection by fluorescence-activated cell sorting analysis. The cells were seeded onto the 6-well plate at a density of $5x10^5$/well in 2 ml and cultured for 24 h. The cells of each well were treated separately and collected after another 12 h of normal culture. The cells were resuspended in 1X binding buffer after washing with ice-cold phosphate-buffered saline (PBS) twice and adjusted to the density of $1x10^6$/ml. Cell suspensions (100 μl) were transferred to a 5-ml tube for fluorescence-activated cell sorting (FACS). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit 5 μl each, were added into cell suspensions and incubated for 15 min at room temperature with avoiding light. The cell mixture was incubated on ice after adding 400 μl of 1X binding buffer, and FACS analysis was conducted within 1 h. $FITC^-/PI^-$ was defined as normal cells, $FITC^+/PI^-$ was defined as apoptotic cells at an early stage, $FITC^+/PI^+$ was defined as apoptotic cells at a late stage, and $FITC^-/PI^+$ was defined as necrotic cells. The experiment was repeated three times, and the apoptotic index (AI) and necrotic rate were calculated using the average measurements. AI = (number of apoptotic cells at a late stage + number of apoptotic cells at an early stage)/total number of cells; necrotic rate = number of necrotic cells/total number of cells.

Transwell assay. The cells at the logarithmic growth phase were seeded onto the 24-well plate at a density of $5x10^4$/well in 2 ml and cultured for 24 h. The normal culture was continued for 24 h after treatment of each group, and the cells were suspended in a serum-free medium after washing twice with PBS. Cells (100 μl) were seeded into the Transwell chamber at a density of $2x10^5$/ml in triplicate format and continuously cultured for 24 h before removing the matrix gel as well as the cells in the upper chamber; the Transwell cells were counted under a microscope using Giemsa staining.

Protein expression detection by western blot analysis. The cells were continuously cultured for 12 h after treatment, and $1x10^7$ cells were collected. The RIPA lysis buffer (150 μl) was added, and the supernatants were used for total protein determination. The protein concentration ranged between 1.5 and 2.5 mg/ml. The supernatants were aliquoted into 20 μl X2 and stored at -80˚C. Sodium dodecyl sulfate (SDS) (10%) polyacrylamide separating gel and 6% concentrating gel were

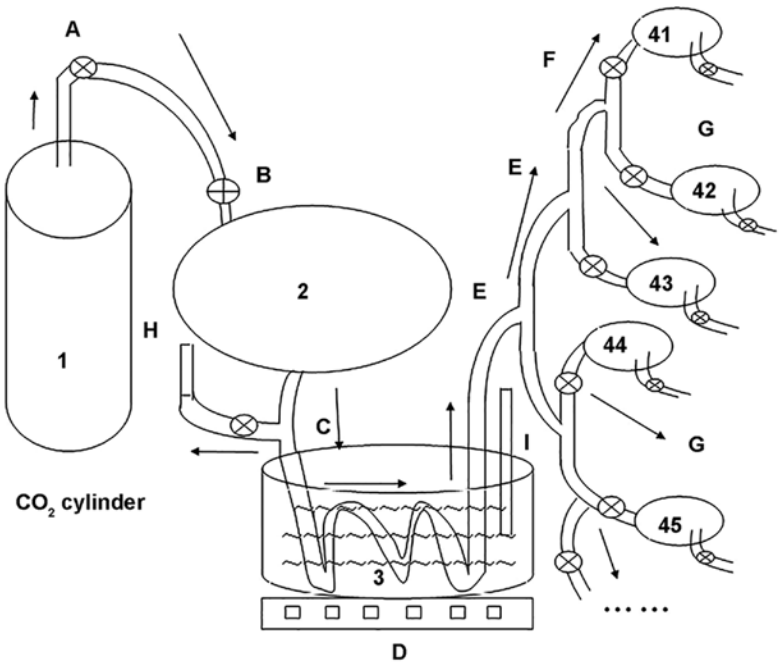

Figure 1. Device to simultaneously warm several mice in CO_2 pneumoperitoneum research, sketch map 1. Medical CO_2 cylinder 2. The disposable urline collection bags 3. Smart heater 41, 42, 43, 44 and 45. Mouse abdominal cavity A. The gas control valve B. Urine collection bags inlet switch C. Medical three-way switch D. Temperature control switch E. Medical three-way switch F. Venous indwelling needle casing inlet switch G. Venous indwelling needle casing gas switch H. Pressure gauge I. Thermometer.

prepared; 80-μg proteins from each group were mixed with 5X SDS loading buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) after denaturing at 100˚C for 10 min. The PAGE was conducted until the dye and protein markers migrated to the desired position. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane. The first antibody was incubated with the PVDF membrane overnight after blocking with skimmed milk solution for 1 h, and the secondary antibody was incubated for 1 h at room temperature before developing the enhanced chemiluminescence blot and photographing. The molecular weight of the targeted protein was estimated using the protein marker, and β-actin was adopted as internal control to evaluate the total amount of protein loaded onto each lane. The images were analyzed using the ImageJ software; the amount of targeted protein = relative gray scale x area (mm^2). The expression level of HSP-70, caspase-3, HIF-lα and MMP-9 was calculated separately for comparison.

Fluorescence quantitative PCR. The cells were collected after each treatment, total RNA was extracted using TRIzol reagent, and cDNA was synthesized using a First Strand cDNA synthesis kit. The primers were designed and synthesized by Sangon Biotech as follows: HSP-70 forward, TACTGTGGACCTG CCAATCG and reverse, TAGCATCATTCCGCTCCTTC; HIF-1α forward, GCAGCAACGACACAGAAACT and reverse, AGCGGTGGGTAATGGAGAC; MMP-9, forward, CCAAC TACGACACCGACGAC and reverse, TGGAAGATGAATGG AAACTGG; caspase-3 forward, AGATGGTTTGAGCCTG AGCA and reverse, CAGTGCGTATGGAGAAATGG; β-actin forward, GATGCAGAAGGAGATCACTG and reverse, TAGT CCGCCTAGAAGCATTTG. The specific primers and Maxima SYBR-Green/ROX qPCR Master were mixed for quantitative PCR (qPCR), with the reaction conditions as follows: 50˚C pretreated for 2 min, 95˚C pre-denaturing for 10 min, 95˚C denaturing for 15 sec, and 60˚C annealing and extension for 60 sec for a total of 40 cycles. Triplicate wells were used, and β-actin was the internal control. Quantitation was represented by cycle threshold value (Ct value). Relative mRNA value = $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{target\ gene}-Ct_{\beta\text{-}actin}$).

In vivo tumor growth and metastasis assay. Logarithmic growth of SW-480 cells were made equal $1x10^7$ cells/l into single cell suspension. The nude mouse abrosia for 1 day were aerosol anesthesia with B halothane, disinfected abdominal skin was cut a 0.8 cm incision in the left lower abdomen, then the cecum, sucked up 0.1 ml cell suspension with OT needle, injected into cecum subserosal, *in situ* colon cancer modelwas established. After 4 weeks, the model mice bearing a tumor were administered aerosol anesthesia again, and a different pneumoperitoneum intervention was established. We applied the in-house device many for many mice to simultaneously warm in the CO_2 pneumoperitoneum experiment and 5-FU intraperitoneal chemotherapy, 5-FU 25 mg/kg, pneumoperitoneum duration of 1 h, at pressure of 12 mmHg. The model mice were sacrificed at 6 weeks. We observed in each group, transplantation tumor weight and viscera metastasis through celiotomy, tumor inhibitory rate = (treatment group weight-control group weight)/control group weight x100%.

Statistical analysis. The data are presented as mean ± standard deviation (SD), and the differences between two groups were analyzed using χ^2 test. The differences among groups were analyzed using one-way analysis of variance test. SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used in the present study and $P<0.05$ was considered as statistically significant.

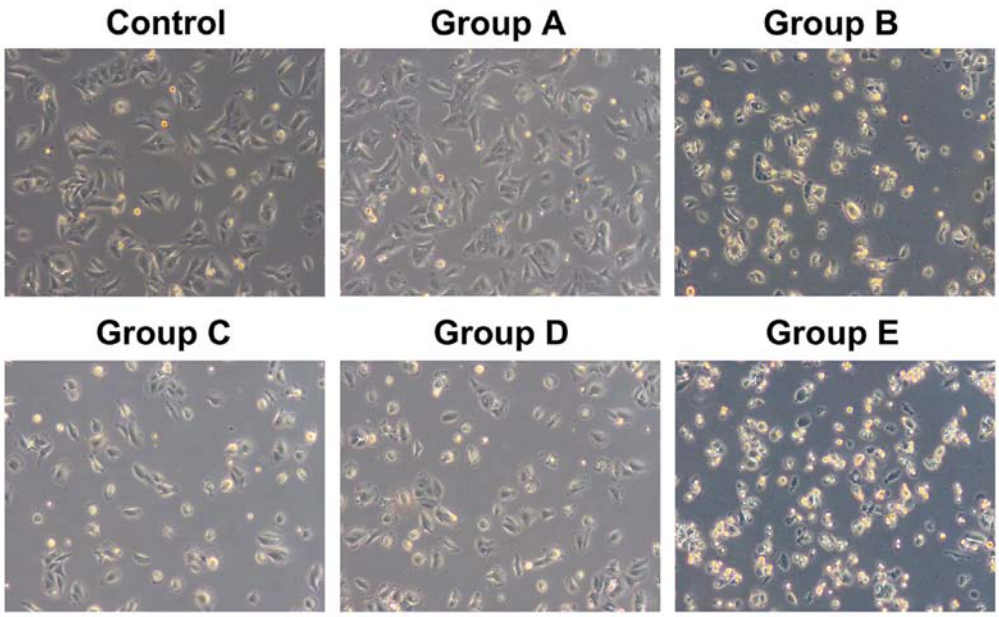

Figure 2. Cell morphology alteration of each group after 24-h treatment.

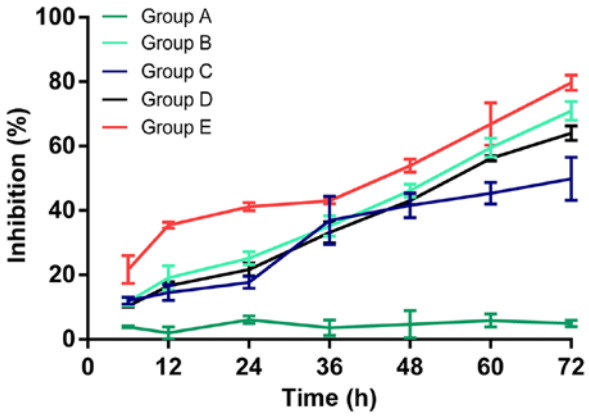

Figure 3. Cell proliferation inhibition in each treatment. Group A, CO_2 pneumoperitoneum; group B, hyperthermic CO_2 pneumoperitoneum; group C: 5-FU; group D, CO_2 pneumoperitoneum + 5-FU; group E, hyperthermic CO_2 pneumoperitoneum + 5-FU.

Results

Dynamic observation of the cell proliferation and morphology under a microscope. The cells from the control group and group A, with rod-like, spindle-like, leave-like or branch-like morphology, were attached to the wall of the petri dish. All cells were in good condition with a rapid growth rate, and reached 100% confluence within 3 or 4 days, without obvious dead cells. However, the majority of cells from groups B and E started to shrink after 12 h and presented with triangular or round morphology without parapodium. The refraction of cells increased; they were detached from the wall of the petri dish and suspended into the culture medium. The cell death and cell debris could be observed after 24 h. The total cell number was reduced and normal morphology loss was aggravated with time. The dead cells and cell debris filled the whole petri dish after 48 h in group E, whereas the cell morphology was not significantly altered within 48 h in groups C and D. The shrinkage and detachment of cells from the petri dish were observed after 48 h, and necrosis and cell debris of small portion of cells were found after 72 h (Fig. 2).

Detection of cell proliferation inhibition by CCK-8 test. The cell proliferation inhibition of each group detected by CCK-8 is shown in Fig. 3. The inhibition rate was 2.05±1.80, 6.16±1.16, 4.72±4.23 and 4.96±1.01% in group A; 19.16±3.77, 25.18±2.07, 46.19±2.00 and 71.00±2.97% in group B; 14.52±2.42, 17.67±1.87, 41.60±3.87 and 49.85±6.73% in group C; 16.58±1.26, 21.69±2.03, 43.09±1.31 and 64.01±2.28% in group D; and 35.49±0.93, 41.18±1.24, 53.96±2.02 and 79.68±2.35% in group E, for 12, 24, 48 and 72 h, respectively. Compared with the control group, no obvious alteration in cell proliferation was observed in group A, whereas significant cell proliferation inhibition was observed in groups B, D, D and E ($P<0.05$); the strongest inhibition was observed in group E compared with groups C and D ($P<0.05$), indicating that hyperthermic CO_2 pneumoperitoneum could reinforce the inhibitory effect of 5-FU on cell proliferation.

Detection of cell apoptosis by FACS analysis. The apoptosis of cells after treatment for 12 h was analyzed using FACS with Annexin V/PI-staining. The rate of apoptosis was calculated using the following formula: apoptosis rate (%)=R3+R5 (Fig. 4). The apoptosis rate was 11.37±0.87, 13.26±0.95, 27.45±1.14, 29.73±0.88, 36.61±0.51 and 65.20±3.11% in the control group and groups A, B, C, D and E, respectively. No significant difference in the apoptosis rate was observed between the control group and group A ($P>0.05$), while apoptosis significantly increased in groups B, C, D and E ($P<0.05$); the most significant apoptosis was observed in group E ($P<0.05$), indicating that hyperthermic CO_2 pneumoperitoneum could enhance the apoptosis induced by 5-FU.

Effect on cell invasion. The invasion of cells was tested by Transwell assay (Fig. 5). The Transwell cell number was 243.7±14.0, 354.2±17.0, 84.5±9.7, 105.7±9.2, 126.6±8.8 and

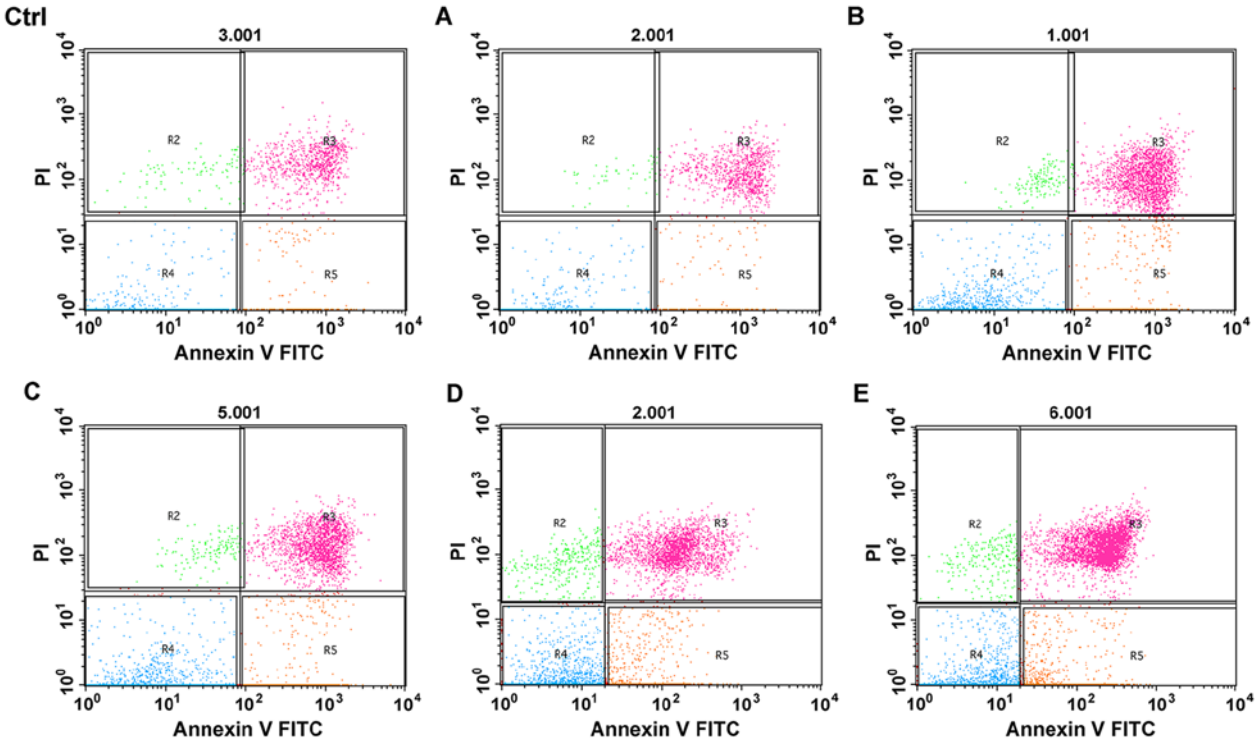

Figure 4. Scattered spots of apoptotic cells stained by Annexin V/PI and processed by FACS.

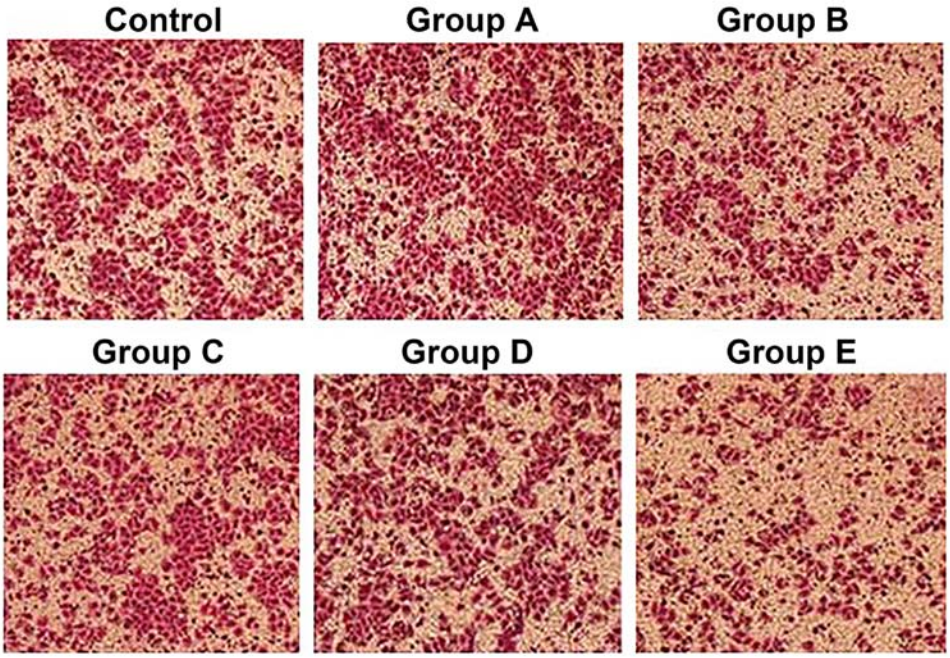

Figure 5. Cell invasion by Transwell assay.

46.2±7.13 for the control group and groups A, B, C, D and E, respectively. Compared with the control group, the Transwell cell number decreased in groups B, C, D and E, but not in group A; the decrease was most significant in group E ($P<0.05$), indicating that hyperthermic CO_2 pneumoperitoneum and 5-FU chemotherapy both have inhibitory effect of cell invasion, hyperthermic CO_2 pneumoperitoneum was able to strengthen the inhibition of cell invasion induced by 5-FU.

Detection of protein expression by western blot analysis. The western blot analysis is shown in Fig. 6 (statistical table.xlsx). Compared with the control group, the expression level of HSP-70 and caspase-3 protein remained unchanged and the expression level of HIF-1α and MMP-9 proteins increased in group A. The expression of caspase-3, HSP-70 and HIF-1α increased and the expression of MMP-9 protein decreased in groups B and E. The expression of caspase-3 increased,

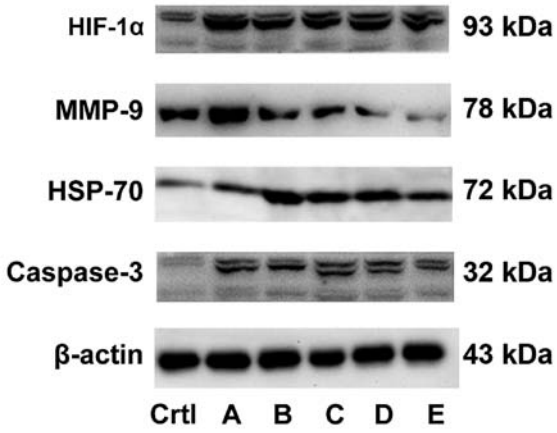

Figure 6. Protein expression alteration by each treatment.

the expression of MMP-9 decreased, and no change in the expression of HIF-1α and HSP-70 was observed in group C. The expression of HIF-1α and caspase-3 increased, the expression of MMP-9 decreased, and no change in the expression of HSP-70 was observed in group D, indicating that the combined effect of hyperthermic CO_2 pneumoperitoneum and 5-FU could promote cell apoptosis by upregulating the expression of HIF-1α, HSP-70 and caspase-3 and inhibit cell invasion by downregulating the expression of MMP-9.

Detection of mRNA level by RT-PCR. The RT-PCR result of each targeted gene 12 h after treatment is shown in Fig. 7 (statistical table.xlsx). The relative expression of HSP-70/β-actin in the control group and groups A, B, C, D and E was 5.40±0.18, 5.84±0.13, 7.51±0.19, 5.55±0.15, 5.73±0.13 and 7.95±0.15,

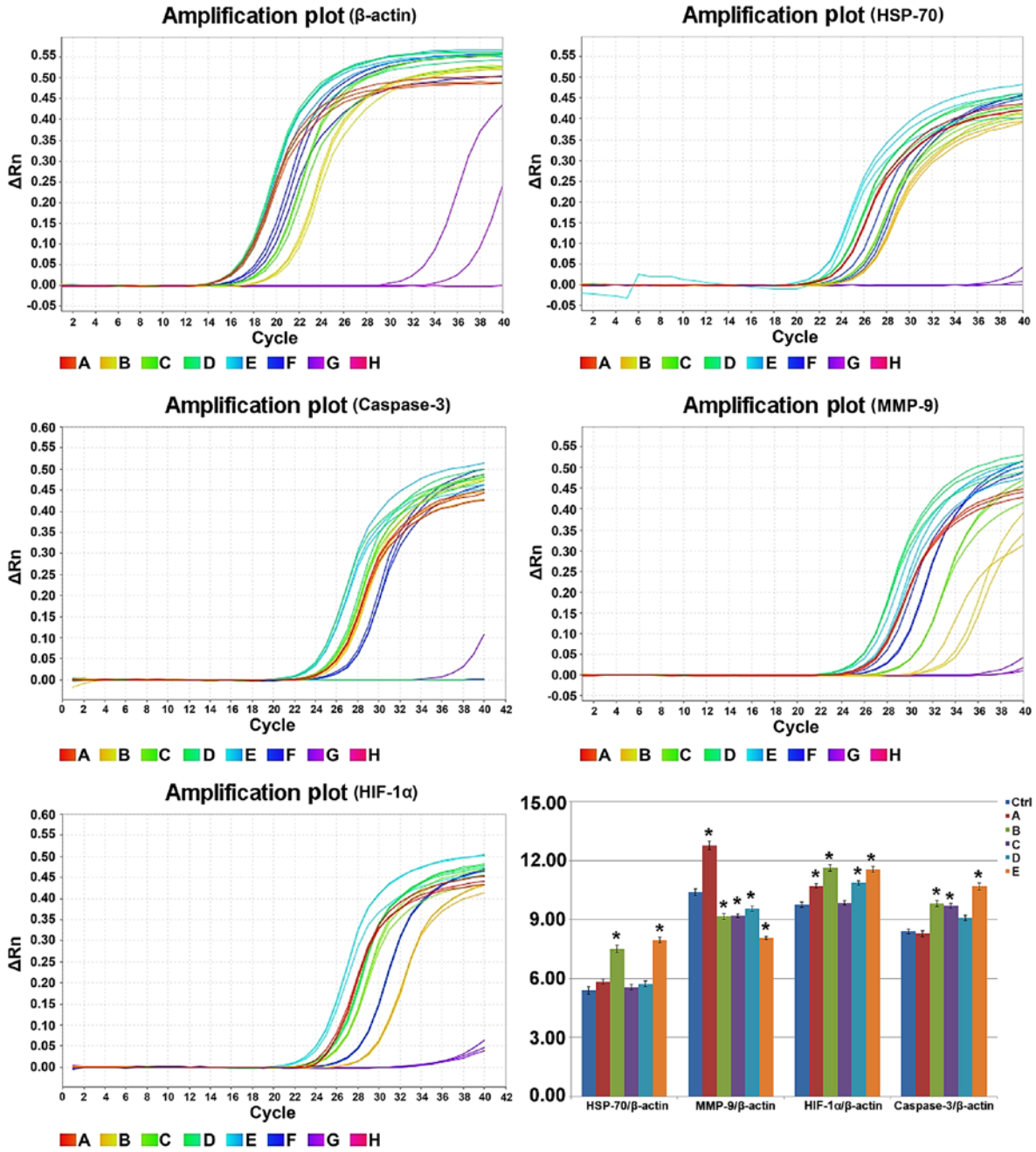

Figure 7. Expression of mRNA coding for each group affected by different treatment.

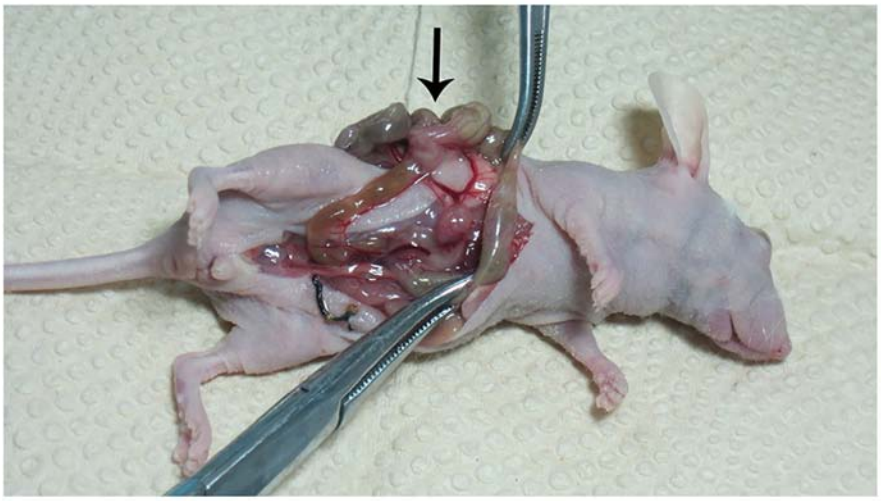

Figure 8. A nude mouse cecum orthotopic transplantation tumor.

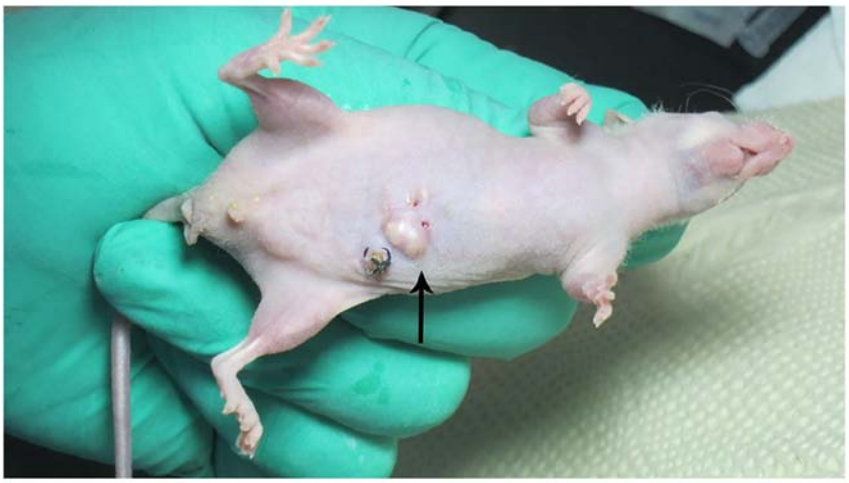

Figure 9. The model nude mouse abdominal wall tumor metastasis.

respectively. The relative expression of MMP-9/β-actin was 10.39±0.17, 12.76±0.22, 9.16±0.15, 9.19±0.09, 9.55±0.13 and 8.07±0.08. The relative expression of HIF-1α/β-actin was 9.75±0.12, 10.70±0.13, 11.63±0.17, 9.85±0.12, 10.86±0.12 and 11.55±0.14. The relative expression of caspase-3/β-actin was 8.40±0.12, 8.28±0.14, 9.81±0.16, 9.70±0.12, 9.08±0.13 and 10.68±0.18. Compared with the control group, the expression level of HSP-70 and caspase-3 mRNA remained unchanged, while the expression level of HIF-1α and MMP-9 mRNA increased in group A ($P<0.05$). However, the expression level of caspase-3, HSP-70 and HIF-1α mRNA increased, while the expression level of MMP-9 decreased in groups B and E. The expression level of caspase-3 mRNA increased while that of MMP-9 mRNA decreased, and no change was observed in the expression level of HIF-1α and HSP-70 mRNA in group C. The expression level of HIF-1α and caspase-3 mRNA increased while that of MMP-9 mRNA decreased, and no change was found in the expression level of HSP-70 mRNA in group D. The present findings indicated that the combined treatment of hyperthermic CO_2 pneumoperitoneum and 5-FU was able to promote cell apoptosis by upregulating the expression of HIF-1α, HSP-70 and caspase-3 and inhibited cell invasion by downregulating the expression of MMP-9.

In vivo tumor growth and metastasis. Of the 72 nude mice 55 cecum vaccination nodular lesions were seen (Fig. 8), diameter from 1.0 to 9.0 mm, the number from 1 to several. The tumorigenic success rate was 76.4% (55/72), 30 nude mice had liver, abdominal wall (Fig. 9), spleen, kidney, gastrointestinal metastases. Viscera metastasis rate was 41.7% (30/72). The number of tumorigenic success in the control group and groups A, B, C, D and E was 10, 11, 8, 9, 9 and 8. The weight of tumor was 0.76±0.05, 0.84±0.06, 0.67±0.06, 0.65±0.05, 0.74±0.05 and 0.45±0.03 g. Compared with the control group, the tumor inhibition rate of groups A, B, C, D and E was 110.5, 88.2, 85.5, 97.4 and 59.2%. Metastasis rate was 70, 72.7, 50, 44.4, 55.6 and 25%. Compared with the control group, the tumor weight and metastasis rate decreased in groups B, C, D and E, but not in group A. The most decrease was seen in group E, indicating that hyperthermic CO_2 pneumoperitoneum and 5-FU chemotherapy both possess inhibition of tumor growth and metastasis, hyperthermic

CO_2 pneumoperitoneum was able to reinforce the inhibition induced by 5-FU.

Discussion

Laparoscopy has been widely used since its adoption for the first time in colorectal surgery in 1991, and has been an important surgical option in colorectal cancer treatment. CO_2 is commonly used to create pneumoperitoneum in laparoscopic surgeries. A concern exists among some surgeons that CO_2 pneumoperitoneum may be associated with tumor cell migration and invasion. The potential mechanisms have been suggested to be the velocity and pressure created by CO_2 pneumoperitoneum-induced tumor cell detachment and spread (6), tumor cells seeded at the puncture site of casing pipes (7), aerosol dissemination of tumor cells by ultrasonic knife, peritoneal acidic hypoxic microenvironment created by CO_2 pneumoperitoneum (8), and cellular immunity alteration (9). However, other researchers believed that CO_2 pneumoperitoneum had no obvious effect on tumor invasion and metastasis (10). Therefore, it was necessary to avoid any possibility of tumor invasion and metastasis caused by CO_2 pneumoperitoneum. Hyperthermia is a novel therapeutic method, which increases temperature systematically or locally to treatment temperature (42-45˚C) to eliminate tumor cells by altering cell signaling pathway or gene network transduction. The major mechanisms are to impair the checkpoint of cell cycle and DNA replication, alter microenvironment, and activate transcriptional factors for lysosomal enzymes, which eventually lead to tumor cell necrosis or apoptosis (11-13). In addition, CO_2 gas is a carrier with good heat conduction and dispersion, which could conduct heat rapidly within the abdominal cavity. Furthermore, 5-FU is the first-line drug for colorectal carcinoma. Marked killing effect on tumor cells could be achieved by using 5-FU as intraperitoneal chemotherapy with high local concentration and prolonged action time. Intraoperative IHCP could inhibit tumor cell migration and eliminate the free tumor cells and micrometastases during operations, thus preventing or reducing tumor invasion and metastasis (14). However, most of IHCP application and studies were conducted in open surgeries; hence, information was lacking on the use of IHCP in laparoscopic operations. Peng *et al* discovered that hyperthermic CO_2 pneumoperitoneum could inhibit colon cancer cell proliferation (15). In the present study, a simulated laparoscopic operation combined with hyperthermic CO_2 pneumoperitoneum and 5-FU intraperitoneal chemotherapy was performed to observe the effect on colon cancer *in vitro* and *in vivo*.

Hyperthermic CO_2 pneumoperitoneum reinforces the inhibitory effect of 5-FU on colon cancer cell proliferation. The inhibitory effect of hyperthermic CO_2 pneumoperitoneum and 5-FU on colon cancer cells was observed under a microscope and using the CCK-8 test. The combination of hyperthermic CO_2 pneumoperitoneum and 5-FU demonstrated the strongest inhibition. The apoptosis of colon cancer cells induced by either hyperthermic CO_2 pneumoperitoneum or 5-FU, or combined treatment was observed by FACS analysis; the combined treatment had the most significant effect. It showed that hyperthermic CO_2 pneumoperitoneum and 5-FU chemotherapy both inhibited tumor growth, and the combined treatment had the most significant effect *in vivo*. The present findings indicated that hyperthermic CO_2 pneumoperitoneum could reinforce the effect of 5-FU by inhibiting cell proliferation and inducing apoptosis. HSP-70 is a molecular chaperone involved in protein synthesis, processing, folding, and transportation, and related to the occurrence, development, drug resistance, and prognosis of tumors (16). Hyperthermic CO_2 pneumoperitoneum alone or combined with 5-FU was shown to upregulate the expression of HSP-70 gene and protein in the present study, which could promote tumor cell apoptosis and necrosis. Caspase-3 is a key member participating in the signaling pathways of apoptosis; it is activated in the early stage of apoptosis, to degrade the substrates in cytoplasm and nuclei, eventually leading to apoptosis (17). Caspase-3 gene and protein were both increased after hyperthermic CO_2 pneumoperitoneum, or 5-FU, or their combined treatment in this study, which could initiate apoptosis and promote cell death. The expression of HIF-1α could be increased under hypoxic conditions, to sustain high energy metabolism and promote angiogenesis by regulating the expression of multiple transcriptional factors and coping with hypoxia, hence promoting tumor invasion, metastasis, and drug resistance (18). In the present study, HIF-1α gene and protein both increased after hyperthermic CO_2 pneumoperitoneum, or 5-FU, or their combined treatment, and it was speculated that transcriptional factors were modulated by an increased level of HIF-1α to promote cell apoptosis and inhibit tumor invasion. The findings indicated that hyperthermic CO_2 pneumoperitoneum and 5-FU combined treatment could promote cell apoptosis by upregulating the expression of HSP-70, HIF-1α and caspase-3.

Hyperthermic CO_2 pneumoperitoneum reinforces the inhibitory effect of 5-FU on colon cancer cell invasion. The inhibitory effect of either hyperthermic CO_2 pneumoperitoneum or 5-FU on colon cancer cell invasion was observed by Transwell assay, and the reinforced effect was achieved by the combined treatment. It showed that hyperthermic CO_2 pneumoperitoneum and 5-FU chemotherapy both inhibited tumor metastasis and the combined treatment had the most significant effect *in vivo*. MMP-9 is a kind of zinc ion-dependent endopeptidase degrading fibrinogen type IV, the major component of extracellular matrix (ECM), leading to tumor invasion and metastasis. MMP-9 has been considered as a marker of tumor invasion and metastasis (19,20). In the present study, the expression of MMP-9 decreased by hyperthermic CO_2 pneumoperitoneum, or 5-FU, or their combined treatment, leading to inhibition of ECM degradation and cancer cell invasion.

In conclusion, the hyperthermic CO_2 pneumoperitoneum reinforced the inhibitory effect of 5-FU on colon cancer cell proliferation and invasion, by upregulating the expression of HSP-70, HIF-1α and caspase-3 at both mRNA and protein levels, downregulating the expression of MMP-9.

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