SPANDIDOS PUBLICATIONS

Hyperthermic CO_2 pneumoperitoneum induces apoptosis in human colon cancer cells through Bax-associated mitochondrial pathway

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Abstract. Peritoneal carcinomatosis of colorectal cancer is common and associated with poor prognosis, which poses a serious challenge and satisfactory treatments are urgently needed. Hyperthermic CO_2 pneumoperitoneum (HT-CO_2) is a new strategy. This study was designed to determine the potential of HT-CO_2 against colorectal cancer cells. Based on an *in vitro* HT-CO_2 study model, the anti-tumor efficacy of HT-CO_2 (42-44˚C for 2-4 h) on human colon cancer COLO 205 cells was evaluated and the mechanisms of actions were analyzed. We found that HT-CO_2 (43-44˚C for 2-4 h) significantly decreased cell viability as determined by WST-8 assay, and the cytotoxicity was attributable to HT-CO_2-induced hyperthermia and extracellular acidification. Apoptosis was the major form of cell killing as demonstrated by Annexin-V/PI flow cytometry and morphological analysis (Hoechst/PI fluorescence microscopy and transmission electron microscopy). Further Western blot analysis and flow cytometric analysis of mitochondrial membrane potential showed that Bax-associated mitochondrial apoptotic pathway played critical role in the induction of apoptosis. We conclude that HT-CO_2 has significant cytotoxic effect on colon cancer cells through induction of Bax-associated mitochondrial apoptosis, and the cytocidal effect is attributable to HT-CO_2-induced hyperthermia and extracellular acidifications. Our data suggest that HT-CO_2 may serve as a potential candidate for treating and/or preventing peritoneal carcinomatosis of colorectal cancer and further investigations both *in vitro* as well as *in vivo* in animal models are needed.

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Abbreviations: HT-CO_2, hyperthermic CO_2 pneumoperitoneum; WST-8,2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

Key words: colorectal cancer, peritoneal carcinomatosis, pneumoperitoneum, hyperthermia, apoptosis

Introduction

Peritoneal carcinomatosis is common in colorectal cancer with high incidence of 10-15% at primary cancer resection and accounts for 25-35% of recurrences (1). Prognosis of patients with peritoneal carcinomatosis is very poor with the median survival varying from 5.2 to 7 months (2,3). Current treatment of peritoneal carcinomatosis often involves two approaches. Systemic chemotherapy coupled with palliate surgery is traditionally used but offers very limited survival benefits. Hyperthermic intraperitoneal chemotherapy (HIPEC) combined with cytoreductive surgery (CRS) can remarkably improve the patients' survival. However, as an aggressive treatment, it is associated with significant treatment-related mortality and morbidity (4,5). These facts pose a serious challenge and warrant urgent need for development of novel strategies.

In this study, we investigated a new strategy for peritoneal carcinomatosis in colorectal cancer: hyperthermic CO_2 pneumoperitoneum (HT-CO_2). CO_2 pneumoperitoneum is used in laparoscopic surgery for creation of operative field. During laparoscopic operations, CO_2 is continually insufflated into peritoneal cavity to keep it extended for operative manipulations. We hypothesize that if the insufflated CO_2 is heated to hyperthermic temperature (42-44˚C) and form hyperthermic CO_2 pneumoperitoneum, it might be able to kill colorectal cancer cells and thus treat the peritoneal carcinomatosis. Hyperthermia is a well documented useful tool for treatment of malignancy including colorectal cancer (6-10). HT-CO_2, as a special heat carrier, can efficiently deliver heat and exert hyperthermia on colorectal cancer cells because of its capability of quick heat dissemination and exchange. Moreover, CO_2 pneumoperitoneum has remarkable impact on extracellular environment, which may augment the effect of

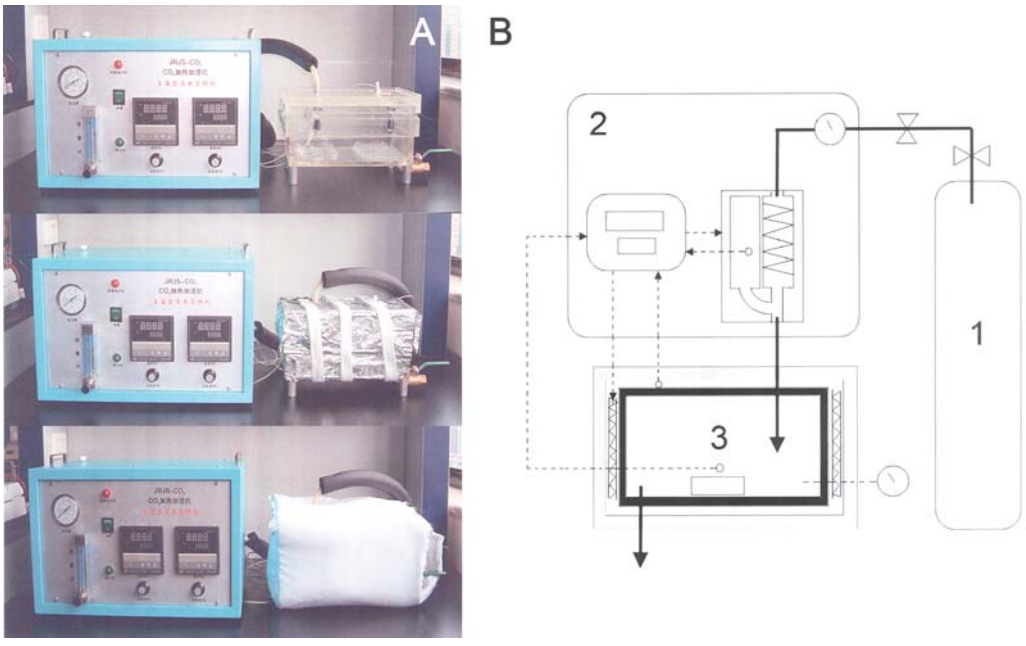


Figure 1. *In vitro* hyperthermic CO_2 pneumoperitoneum study model: (A) model in different status. From the top down, the affiliated heater and muff of pneumoperitneum chamber are added in turns. The bottom one is ready for experiment; (B) schematic illustration of the model: CO_2 released from CO_2 container (1) enters the CO_2 heater and humidifier (2). After being heated and humidified to intended temperature and humidity, it is insufflated into the pneumoperitoneum chamber (3) which has been preheated to 37˚C (to simulate human peritoneal wall) with its affiliated heater and muff. Colon cancer cells loaded in plates are placed inside the chamber. Under regulation of control system of CO_2 heater and humidifier (1), the cells are exposed to hyperthermic CO_2 pneumopeirtoneum at constant temperature (42-44˚C), pressure (15 mmHg), flow rate (10 l/min) and humidity (>95%) with accurate stability.

hyperthermia aforementioned. For example, CO_2 pneumoperitoneum can decrease extracellular pH and induce hypoxia and oxidative stress, whereas extracellular acidification and oxidative stress can significantly enhance cytotoxicity of hyperthermia as documented (11-17). In addition, HT-CO_2 combines with laparoscopic surgery. Laparoscopic surgery for colorectal cancer has been applied increasingly recently and demonstrated being able to offer equivalent safety and curative effect with advantages of morbidity and mortality compared to traditional open surgery (18). Therefore, HT-CO_2 is a promising strategy which might be able to achieve considerable anti-tumor efficacy with minimal morbidity and mortality.

This study was designed to *in vitro* evaluate the potential of HT-CO_2 against peritoneal carcinomatosis in colorectal cancer. We created an *in vitro* HT-CO_2 study model to simulate the hyperthermic CO_2 pneumoperitoneum in human body. Based on the model, the anti-tumor effects of HT-CO_2 on colorectal cancer cells and the underlying mechanisms of actions were investigated for the first time.

Materials and methods

Cell lines. Human colon cancer COLO 205 cells (ATCC, USA), originated from peritoneal fluid of patients with colon cancer, were used in our study to represent peritoneal carcinomatosis of colorectal cancer. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, UK), and maintained at 37˚C in a humidified 95% atmosphere containing 5% CO_2.

In vitro HT-CO_2 study model. An *in vitro* HT-CO_2 study model (Fig. 1) was created to simulate the hyperthermic CO_2 pneumoperitoneum in human body (the model has applied patent protection from State Intellectual Property Office, P.R. China, Patent. Nos. 200620047773.6, 200620047772.1, 200610118324.0). Structure and principles of the model are illustrated in Fig. 1B. The model enables the exposure of cells to HT-CO_2 at constant temperature, pressure, flow rate and humidity with accurate stability of ±0.3˚C, ±1 mmHg, ±0.5 l/ min, and ±5%, respectively.

Experimental design. Exponentially growing cells were seeded in 96/6-well plates for experiments. The cells were divided into 4 groups and treatments were as follow: i) hyperthermic CO_2 pneumoperitoneum (HT-CO_2): the cells were treated with the *in vitro* HT-CO_2 study model and exposed to hyperthermic CO_2 pneumoperitoneum at various temperatures (42-44˚C) with 15 mmHg pressure, 10 l/min flow rate and >95% humidity for 2-4 h; ii) conventional hyperthermia: the cells were incubated at various temperatures (42-44˚C), >95% humidity and 5% CO_2 for 2-4 h, using incubator (Kendro Heraeus, Germany) as described by Yuen *et al* (19); iii) normo-thermic CO_2 pneumoperitoneum: CO_2 at room temperature was insufflated into the pneumoperitoneum chamber (Fig. 1) using Stryker's 40-liter insufflator (Stryker, USA). The cells loaded in plates were placed inside the chamber and exposed to CO_2 at room temperature (25˚C) with 15 mmHg pressure and 10 l/min flow rate for 2-4 h; iv) conventional incubation: the cells were incubated conventionally (37˚C, >95% humidity, 5% CO_2).

lity assay. Cell viability was determined with WST-8 ty assay using Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instructions. Briefly, cells were seeded onto a 96-well microplate at $5x10^3$ cells/well. After various treatment and further incubation for 6 h, 10 μl of the CCK-8 solution was added to each well of the microplate. After another incubation for 4 h, the absorbance at 450 nm was measured by a microplate reader (μQuant, Bio-Tek, USA). Cell viability (%) = (mean treated absorbance/mean untreated absorbance) x 100%.

Analysis of extracellular temperature and pH. Extracellular temperature change in the medium was monitored by thermal probe (PT100). Extracellular pH change was determined with Sartorius Professional-Meter (Sartorius, Germany). To analyze the role of HT-CO_2-induced extracellular acidification in the cytotoxicity of HT-CO_2, acidification inhibition was performed as described by Leng *et al* (20). HEPES (hydroxyethyl piperazine ethanesulfonic acid) (50 mmol/ml) was added to maintain extracellular pH at 7.2-7.3 during the treatment of HT-CO_2. Cell viability between HT-CO_2, HT-CO_2+HEPES and HEPES groups was compared.

Hoechst 33342 and PI fluorescent microscopy. Hoechst 33342 and PI fluorescent microscopy analyses were performed to describe cell death (Hoechst 33342/PI Detection Kit, Keygen Biotech, P.R. China). Briefly, following treatment, the cells were harvested and washed with cold PBS. After resuspension in 1 ml RPMI-1640 medium, Hoechst 33342 (5 μg/ml) was added and incubated for 8 min in dark. Then the cells were centrifuged and resuspended in 1 ml PBS, PI (50 μg/ml) was added. The cells were then mounted onto slides. Images were obtained using a fluorescent microscope (Olympus IX71, Olympus, Japan) excited at 350 and 530 nm for Hoechst 33342 and PI, respectively. The photographs were merged and analyzed by Act-2U software (Nikon, Japan).

Transmission electron microscopy. Cells were harvested, pelleted and pre-fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4˚C for 2 h. After a rinse with PBS, the samples were post-fixed with 1% osmium tetroxide (TAAB Laboratories Equipment, UK) at 4˚C for 2 h, dehydrated with graded ethanol (50, 75 and 95-100%) and then embedded with Epon 618 (Ladd Research Industries Inc., USA). Ultra-thin sections of 50-80 nm were obtained through Leica ultracut R (Vienna, Austria) and stained with lead citrate and uranyl acetate. Then the ultra-thin sections were examined with a Philips CM-120 transmission electron microscope (Philips, The Netherlands) operating at 80 kV.

Annexin V and PI flow cytometry. Annexin V and PI flow cytometry was performed to detect and quantify apoptosis according to the manufacturer's instructions (Annexin V-FITC apoptosis detection kit I, Beckman Dickinson, USA). In brief, following treatment and a further incubation of 12 h, the cells were harvested and washed twice with ice-cold PBS. The cells were then resuspended in 1X binding buffer at a density of $1x10^6$ cells/ml, Annexin V-FITC (5 μl) and PI (5 μl) were added. After incubation for 15 min at room temperature (25˚C) in the dark, the cells were analyzed with FACS Calibur (Beckman-Coulter, Beckman Dickinson) and the data were analyzed with CellQuest software.

Flow cytometric determination of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\Psi$m) was measured by rhodamine-123 (R123) (Molecular Probes, Sigma, USA) retention flow cytometry. Briefly, the cells ($1x10^6$) were washed twice with ice-cold PBS and incubated with R123 (1 μg/ml) at 37˚C for 5 min. PI (10 μg/ml) was added to identify dead cells. R123 fluorescence intensity was analyzed with a FACS Calibur flow cytometer using Cellquest software (Beckman Dickinson) and the mean fluorescence intensity of 10,000 cells was calculated for each sample.

Western blot analysis. Cells were harvested at various time in succession (0/1/2/4/8/16/24 h) after treatment. Total and cytosolic protein were extracted using M-PER™ Mammalian Protein Extraction Reagent (Pierce, USA) and Nuclear Extract Kit (Active Motif, USA), respectively. Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce). Antibodies against Bax, Bak, Bcl-2, Bcl-xL, p53, cytochrome C (Cyto C), caspases 8 and 9 were purchased from Santa Cruz Biotechnology (CA), whereas antibody against ß-actin was obtained from Sigma-Aldrich. Using serial products of Bio-Rad (USA) according to their instructions, Western blot analysis was carried out to detect the expression of p53, Bcl-2, Bax, Bcl-XL, Bak, ß-actin, procaspase 8/9 and their cleaved fractions in total protein and Cyto C in cytosolic protein. Proteins were visualised using a peroxidase-conjugated anti-mouse/rabbit secondary antibody (Santa Cruz Biotechnology) and Super Enhanced chemiluminescence visualization kit (Applygen Technologies Inc., P.R. China) through Innotech cooled CCD system (Alpha Innotech, USA).

Statistics. The data are expressed as mean ± SD. The differences between various groups were determined by one-way analysis of variance (ANOVA) or two-tailed paired Student's t-test with SPSS 11.5 software. Differences were considered statistically significant at $p<0.01$.

Results

HT-CO_2 resulted in significant inhibition of cell viability of colon cancer cells. We first assessed the effect of HT-CO_2 on the viability of human colon cancer COLO 205 cells. As shown in Fig. 2, HT-CO_2 resulted in a significant decrease in viable cells starting from HT-CO_2 at 42˚C for 4 h ($P<0.01$), suggesting a strong anti-tumor response against human colorectal cancer cells. Moreover, it was also observed that HT-CO_2 was more cytotoxic than conventional hyperthermia when compared at same level of temperature and duration ($P<0.01$, starting from 43˚C for 2 h). After treatments at 44˚C for 4 h, inhibition of cell viability of HT-CO_2 was 57% more than that of conventional hyperthermia. These data indicated that HT-CO_2 indeed had an enhanced potent anti-tumor effect as expected.

HT-CO_2-induced cytotoxicity was attributable to hyperthermia and extracellular acidification. Since HT-CO_2 could exert

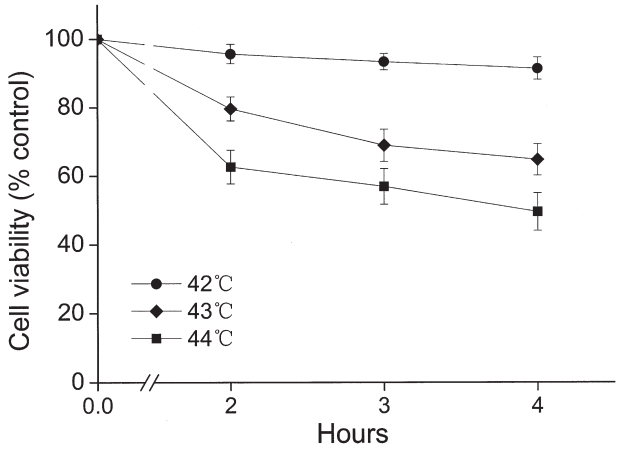


Figure 2. Cytotoxic effect of hyperthermic CO_2 pneumoperitoneum (HT-CO_2) on colon cancer COLO 205 cells. Cell viability was measured using WST-8 cytotoxicity assay. Data are presented as mean ± SD of 18 independent values obtained from three separate experiments with 6 replicates for each one.

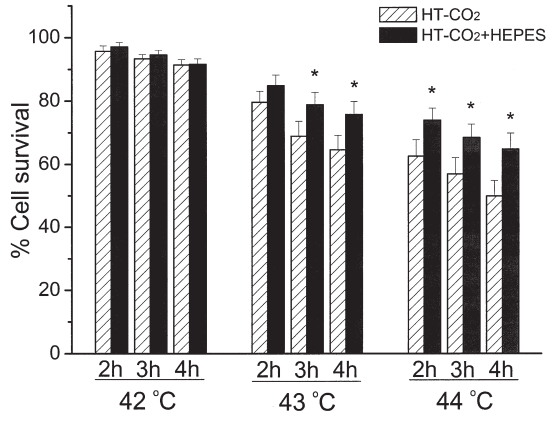


Figure 3. Cytotoxicity of HT-CO_2 was remarkably inhibited following HEPES blocking of the HT-CO_2-induced extracellular acidification (*P<0.01). Cytotoxicity was measured using WST-8 cytotoxicity assay. Data are presented as mean ± SD of 18 independent values obtained from three separate experiments with 6 replicates for each one.

hyperthermia and influence extracellular environment as described in Introduction, we asked whether the HT-CO_2-resulted hyperthermia and extracellular acidification contributed to HT-CO_2-induced cytotoxicity. Analysis of the extracellular temperature and pH change showed that the extracellular temperature reached the same level as that of HT-CO_2 while extracellular pH decreased from 7.4 to 6.7 within 30 min, which demonstrated that COLO 205 cells were actually exposed to a hyperthermic and acidic environment for ~2-4 h. The role of hyperthermia in cytotoxicity of HT-CO_2 was demonstrated by results of conventional hyperthermia which exhibited significant cytotoxic effect on COLO 205 although a high dose was needed (>43˚C for 2 h). To identify the role of extracellular acidification, an acidification inhibition experiment was performed using HEPES and results showed that the cytotoxicity was remarkably decreased after acidification was inhibited (P<0.01, starting from 43˚C for 2 h) (Fig. 3). These findings indicated that the cytotoxicity of HT-CO_2 was attributable to the hyperthermia and extracellular acidification.

HT-CO_2-induced significant apoptosis, which was the major form of cell-killing. To elucidate the mechanism of HT-CO_2-induced cell-killing, we next determined the mode of cell death of COLO 205 cells treated with HT-CO_2. For the purpose, morphological assessment and Annexin-V/PI flow cytometry were used. Following treatment of HT-CO_2, the cells were first subjected to Hoechst 33342 and PI fluorescent microscopy which could distinguish apoptosis from necrosis. Results showed that apoptosis was the major form of cell death although necrosis increased when treated with HT-CO_2 at high temperature for long duration (Fig. 4A-C). A further transmission electron microscopy confirmed the finding, typical apoptosis was observed (Fig. 4D-F). After determination of apoptosis by morphological analysis, Annexin-V and PI flow cytometry was used to quantify the extent of apoptosis. Results of Annexin-V/PI flow cytometry indicated a significant temperature- and duration-dependent induction

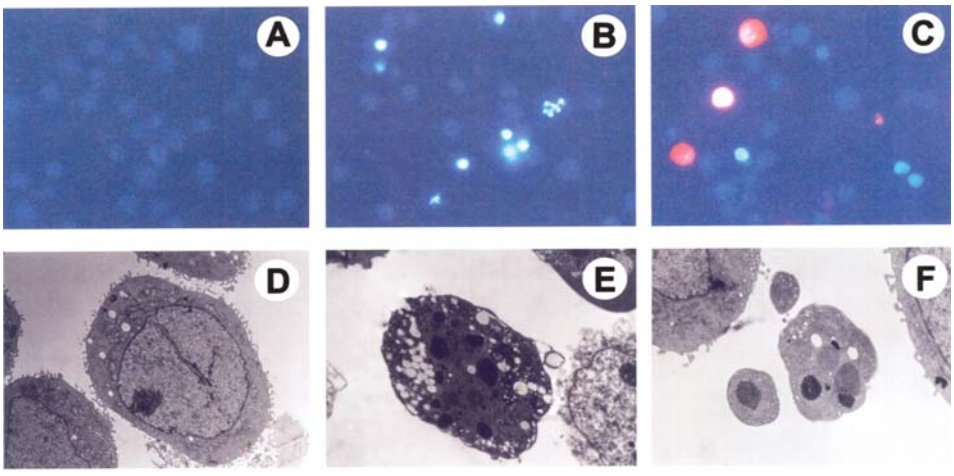
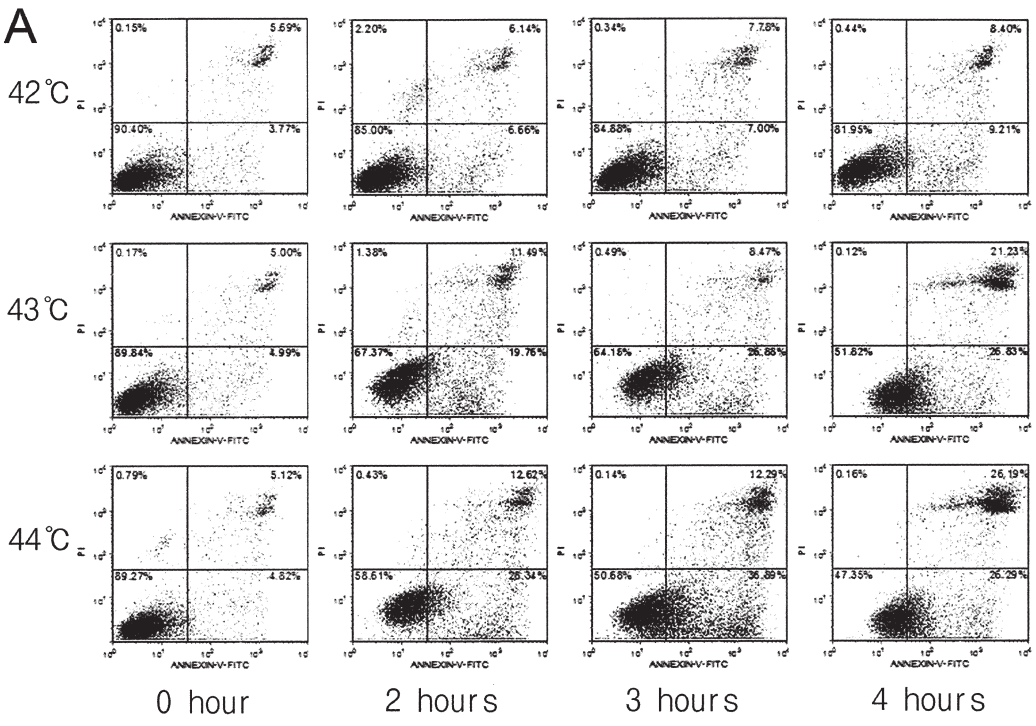


Figure 4. Morphological analysis of HT-CO_2-induced cell-killing: apoptosis was the major form of cell death. (Upper) Hoechst 33342 and PI fluorescent microscopy (x200): (A) normal cells (light blue staining); (B) apoptotic cells (brilliant blue staining with nuclear condensation or fragmentation, HT-CO_2 at 43˚C for 3 h); (C) necrotic cells (shiny red staining with enlarged cell volume) increased when exposed to HT-CO_2 at high dose (HT-CO_2 at 44˚C for 4 h). (Lower) Transmission electron microscopy (x3400): (D) normal cells; (E) apoptotic cells (cell shrinkage with higher density, chromatin condensation and margination, nuclear fragmentation); (F) apoptotic bodies (HT-CO_2 at 43˚C for 3 h). Results are from one experiment that is representative of three similar experiments.



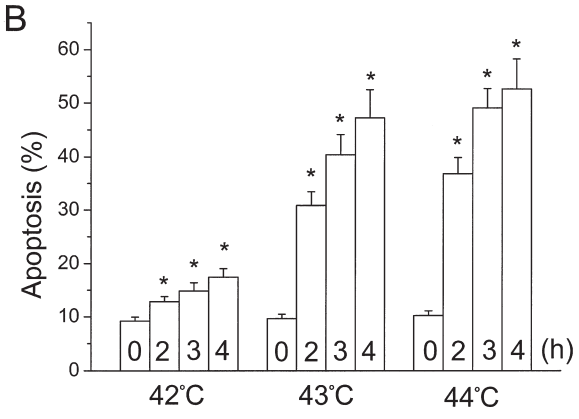


Figure 5. Analysis of Annexin V and PI flow cytometry showed significant temperature- and duration-dependent induction of apoptosis in COLO 205 cells. (A) Representative cytograms of apoptosis induced by HT-CO_2 at various temperature for 2-4 h; (B) Quantitation of apoptosis: the apoptotic cell population was counted as the sum of early apoptotic (Annexin V^+ and PI^-) and late apoptotic/secondary necrotic cells (Annexin V^+ and PI^+). The data are presented as means ± SD of four separated experiments with triplicates for each one ($^*P<0.01$).

of apoptosis (Fig. 5). The percentage of apoptotic cells was significantly increased from 9.6±0.7% (control) to 12.8±1.0% (43˚C for 2 h, minimum) and 52.6±4.6% (44˚C for 4 h, maximum) 12 h after treatment with HT-CO_2.

HT-CO_2-induced apoptosis through Bax-associated mitochondrial mechanism. Since mitochondrial mechanism often plays critical role in hyperthermia-induced apoptosis, experiments were done to assess its role in the HT-CO_2-induced apoptosis. Analysis of mitochondrial membrane potential ($\Delta\Psi m$) by rhodamine-123 retention showed that HT-CO_2 treatment caused significant disruption of mitochondrial membrane potential (Fig. 6A and B). Western blot analysis revealed that cytochrome C was released from mitochondria into cytosol and caspase 9 was activated (Fig. 6C). Further Western blot analysis of Bcl-2 family and p53 proteins showed an increased expression of Bax protein while no increases of Bak, Bcl-2, Bcl-xL and p53 (Fig. 6C). These data indicated that Bax-associated mitochondrial mechanism played a critical role in HT-CO_2-induced apoptosis. In addition, we also examined the activity of caspase 8 which was generally thought as the principal initiator caspase of death receptor apoptotic pathway. No activation of caspase 8 was observed (Fig. 6C), which indicated that the death receptor apoptotic mechanism might not be involved.

Discussion

Peritoneal carcinomatosis in colorectal cancer is associated with poor prognosis. In this study, a new strategy for its management with hyperthermic CO_2 pneumoperinoneum (HT-CO_2), was preliminarily evaluated. Based on an *in vitro* HT-CO_2 study model, the anti-tumor effects of HT-CO_2 on colon cancer cells and its possible mechanisms of actions were investigated for the first time.

Our results demonstrated that HT-CO_2 had significant cytotoxic effect on colon cancer COLO 205 cells, and the cytotoxicity was attributable to HT-CO_2-induced hyperthermia and extracellular acidification. Hyperthermia has been well documented as a cytotoxic agent for malignancy including colorectal cancer (6-10,21,22). Meanwhile, it is also well established that extracellular acidification can significantly enhance the cytotoxicity of hyperthermia and the enhancement effect of acidification was independent of p53 and bcl-2 status (12-14,23-25). Therefore the hyperthermia combined

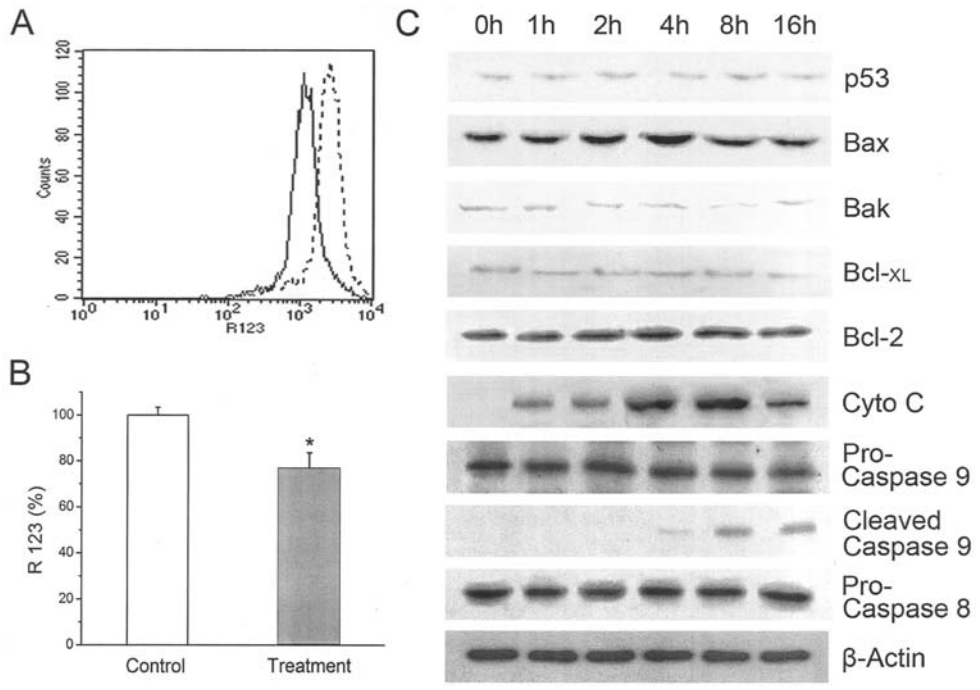


Figure 6. HT-CO_2-induced apoptosis of COLO 205 cells via Bax-associated mitochondrial mechanism, exemplified here by HT-CO_2 at 43˚C for 3 h (A and B). Disruption of mitochondrial membrane potential ($\Delta\Psi m$) after HT-CO_2 treatment, determined by rhodamine-123 (R123) retention assay. The fluorescent intensity of HT-CO_2-treated cells (solid line) significantly decreased compared to that of control (untreated) cells (dotted line) ($^*P<0.01$). (C) Western blot analysis showed up-regulation of Bax protein, release of cytochrome C from mitochondria into cytosol and activation of caspase 9. The results are representative of three separate experiments.

with extracellular acidification was expected to be a new strategy for human cancer because numerous cancers bear mutated p53 and overexpress Bcl-2 (12,14). Ohtsubo *et al* (12) reported that colon cancer cells subjected to hyperthermia (42˚C, 1 h) in pH 6.6 medium resulted in significantly more apoptosis than those exposed to pH 7.5 medium (RKO.C cells: 18 vs. 10%; RC10.1 cells: 34 vs. 16%). In our study, the COLO 205 cells subjected to HT-CO_2 were actually exposed to a hyperthermic (42-44˚C) and acidic environment (pH 6.7) for approximately 2-4 h, significant cytotoxicity was induced and the cell-killing effect of HT-CO_2 was also significantly more than that of hyperthermia alone, which was similar with the aforementioned report.

Moreover, our data also demonstrated that the cytotoxicity of HT-CO_2 was mediated via induction of apoptosis. Apoptosis is well-known as cell death mechanism by which mild hyperthermia (<45˚C) kills cancer cells including colorectal cancer cells, but the efficacy of apoptotic induction is cell line dependent (9,21,22). For example, human colon cancer HT-29 cells subjected to hyperthermia (40˚C, 45 min) shows an apoptotic rate of 100%, whereas HCT-116 cells exposed to hyperthermia (43˚C, 2 h) indicate only an approximately 50% rate (9,22). Furthermore, under acidic condition, cells are more sensitive to hyperthermia-induced apoptosis (12,14,23). Ohtsubo *et al* (12) demonstrated that colon cancer RKO.C and RC10.1 cells treated with hyperthermia under acidic condition (pH 6.6) has significantly increased apoptotic rate compared to that of hyperthermia under pH 7.5. In our study, the COLO 205 cells were exposed to hyperthermic (42-44˚C) and acidic environment (pH 6.7) for approximately 2-4 h, significant temperature- and duration-dependent apoptosis was induced and a maximal apoptotic rate of 52±4.5% was achieved after treatment with HT-CO_2 at 44˚C for 4 h.

Furthermore, analysis of molecular mechanism of HT-CO_2-incuced apoptosis showed that Bax-associated mitochondrial mechanism played a critical role. It was reported that mitochondrial mechanism is often involved in hyperthermia-induced apoptosis (19,26). Yuen *et al* (19) reported that hyperthermia (43˚C, 3 h)-induced apoptosis through mitochondrial mechanism in L929 cells. With use of cyclosporin A to inhibit the dissipation of mitochondrial membrane potential, the apoptosis was significantly suppressed. Our data demonstrated that HT-CO_2-induced apoptosis in COLO 205 cells was also through mitochondrial mechanism and the death receptor apoptotic mechanism appeared not to be involved. In addition, our results showed that Bax was associated with apoptosis. Bax family often regulates mitochondrial apoptosis, but its role in colorectal cancer cells for hyperthermia (with or without extracellular acidification)-induced apoptosis are complicated and appear to be cell line dependent. Sturm *et al* (22) reported that hyperthermia (43˚C, 2 h)-induced apoptosis in HCT116 cells was Bax-dependent, whereas Ohtsubo *et al* (12) reported no involvement of Bax, Bcl-2 in RKO.C and RC10.1 cells subjected to hyperthermia (42˚C, 1 h) with acidification (pH 6.6). In this study, HT-CO_2-induced apoptosis in COLO 205 cells indicated an involvement of Bax, suggested that HT-CO_2 may up-regulate expression of Bax and subsequently trigger the mitochondrial apoptotic pathway.

In conclusion, this preliminary study demonstrated that hyperthermic CO_2 pneumoperitoneum (HT-CO_2) has potent

r effect on colorectal cancer cells through induction sociated mitochondrial apoptosis, and the anti-tumor effect was attributable to HT-CO_2-induced hyperthermia and extracellular acidifications. Our data suggest that HT-CO_2 may serve as a potential candidate for treating and/or preventing peritoneal carcinomatosis in colorectal cancer and further investigations including *in vivo* studies are needed.

Acknowledgments

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