

RESEARCH COMMUNICATION

Antitumor Effects of Hyperthermic CO₂ Pneumoperitoneum on Human Gastric Cancer Cells

Hou-Min Zhou[&], Bo Feng[&], Hong-Chao Zhao, Min-Hua Zheng^{*}

Abstract

Aim: To elucidate the effects of hyperthermic CO₂ pneumoperitoneum on human gastric AGS cells. **Methods:** Based on a newly devised *in vitro* study model, we evaluated the anti-cancer effects of HT-CO₂ (42-44°C for 2-4h) on human gastric cancer cells, and also the corresponding mechanisms. **Results:** HT-CO₂ (42-44°C for 2-4h) severely inhibited cell proliferation as assessed by Cell Counting Kit-8 assay, while inducing apoptosis in a temperature- and time-dependent manner demonstrated by annexin-V/PI flow cytometry and morphological analysis (Hoechst/PI fluorescence). In addition, it was found that HT-CO₂ (42-44°C for 2-4h) promoted the up-regulation of Bax by western blotting. Significantly, it could also suppress gastric cancer cell invasion and metastasis by *in vitro* invasion and motility assay. **Conclusion:** In conclusion, HT-CO₂ had an efficacious cytotoxic effect on gastric cancer cells through Bax-induced mitochondrial apoptotic signaling. Our studies indicate that it may serve as a potential therapy for peritoneal carcinomatosis of gastric cancer. Further investigations *in vivo* using animal models are now urgently needed.

Keywords: Gastric cancer - peritoneal carcinomatosis - pneumoperitoneum - apoptosis - invasion and metastasis

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Introduction

Gastric cancer is the fourth most common cancer and is the second leading cause of cancer-related mortality (Bertuccio et al., 2009). Tumor progression into the peritoneal cavity always indicates a poor prognosis, with death resulting from peritoneal carcinomatosis (PC). Patients with gastric cancer peritoneal carcinomatosis have an extremely poor prognosis with a median survival estimated to be 1-3 months (Sadeghi et al., 2000). Even the patients undergone a potentially curative resection of gastric cancer, they may have 5-20% of the risk of PC. Although great stride has been made in the treatment of gastric carcinomas, no satisfactory therapies are available because of micrometastases and free-floating carcinoma cells already existing in the peritoneal cavity.

The hallmarks of cancer comprised six biological capabilities acquired during the multistep development of human tumors. They included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Growing experimental evidence supported each of the core hallmark capabilities is regulated by partially redundant signaling pathways. Consequently, a target therapeutic agent inhibiting one key pathway in a tumor may not completely shut off a hallmark capability (Hanahan et al., 2011). Hyperthermia had a direct cell-killing effect on tumor cells and induced cell apoptosis

through a series of related genes, including wild-type p53, Fas, bcl2 and other genes. Hyperthermia could also destroy the tumor blood vessels and change the expression of matrix metalloproteinase (MMP) and adhesion molecules to inhibit tumor invasion and metastasis. Therefore, hyperthermia might be a promising therapy on cancer owing to its multiple effects on the hallmarks of cancer. In this study, we designed a novel strategy against peritoneal carcinomatosis in gastric cancer: hyperthermic carbon dioxide (CO₂) pneumoperitoneum (HT-CO₂) (42-44°C for 2-4 hours). Yuanfei Peng et al. reported that HT-CO₂ had potent anti-tumor effect on colorectal cancer cells through induction of Bax-associated mitochondrial apoptosis, and the anti-tumor effect was attributable to HT-CO₂ induced hyperthermia and extracellular acidifications (Peng et al., 2008). However, the effect of HT-CO₂ on human gastric cancer cells are poorly understood. In addition, if such antitumor effect does exist, another aim of this study is to elucidate the molecular mechanism of such effect induced by HT-CO₂.

Materials and Methods

In vitro HT-CO₂ study model

Based on the previous *in vitro* study model (the model has applied patent protection from State Intellectual Property Office, P.R. China, Patent. Nos. 200620047773.6, 200620047772.1, 200610118324.0) designed by our research group, we made some improvements and

Department of Surgery, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai Institute of Digestive Surgery, Shanghai Minimally Invasive Surgery Center, Shanghai, China [&]Equal contributors ^{*}For correspondence: fengbo2022@163.com

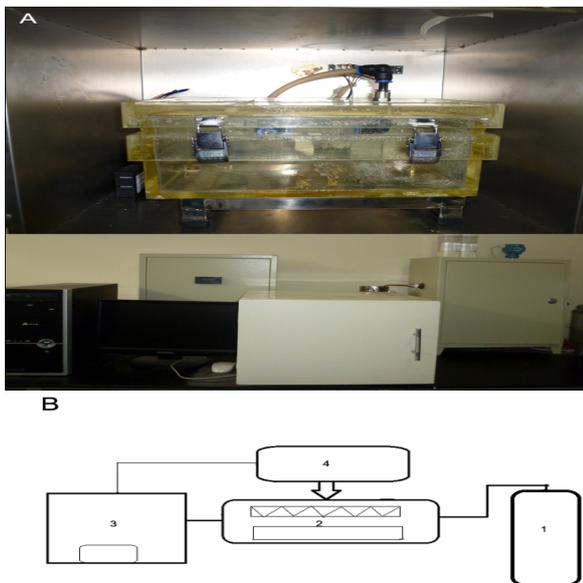


Figure 1. In Vitro Hyperthermic CO₂ Pneumoperitoneum Study Model

innovations (Figure 1A). The upper part of Fig. 1A was artificial pneumoperitoneum. The temperature outside the plexiglass box was maintained at 37°C in order to simulate the human body core temperature. The gastric cancer cells located in the plates were placed inside the plexiglass box exposed to constant temperature (42-44°C), pressure (15mmHg), flow rate (10L/min) and humidity (>95%). The lower part of Figure 1A was the schematic diagram of the overall in vitro study model. The structure and principles are demonstrated in Figure 1B. The carbon dioxide from the container (1) was transported into the heating humidification devices (2) After being heated and humidified to predetermined temperature and humidification, soon after the gas was insufflated into the pneumoperitoneum chamber (3) which had been heated to 37°C in advance so as to imitate the environment of human peritoneal cavity. Human gastric cancer AGS cells implanted into the plates were placed inside the chamber. The whole system was regulated by a computer (4) Using the control software, the signals fed back from the chamber could be processed in time in order to maintain a constant temperature and humidification (the cells were exposed to at a constant temperature at 42-44°C, pressure at 15mmHg, flow rate at 10L/min and humidity >95%).

Exponentially growing cells were seeded in 96/6-well plates. In our study, the treatments were divided into three groups: HT-CO₂, conventional hyperthermia (CH), conventional incubation (CI). HT-CO₂: the cells were treated with the in vitro HT-CO₂ study model and exposed to hyperthermic CO₂ pneumoperitoneum at specific temperatures (42-44°C) with 15 mmHg pressure, 10L/min flow rate and >95% humidity for 2-4h; Conventional hyperthermia(CH): the cells were incubated at various temperatures (42-44°C), >95% humidity and 5% CO₂ for 2-4h; Conventional incubations(CI): the cells were incubated under standard conditions (37°C, >95% humidity, 5% CO₂).

Cell culture

Human gastric carcinoma AGS cell line (obtained

from ATCC; ATCC number; CRL-1739) was originally derived from an adenocarcinoma of the stomach from a 25-year-old Caccasian female, who received no prior therapy. AGS was maintained in RPMI Medium1640. Cells were cultured at 37°C in 5% CO₂ in air in medium supplemented with 10% fetal bovine serum (FBS). Cells were seeded in 96/6 well plates 24hr before treatment.

Cell viability assay

The cell viability was measured by WST-8 cytotoxicity assay with Cell Counting Kit-8. Inoculate cell suspension (200 µl/well) in a 96-well plate at 3x10³ cells /well. After different treatment and extra incubation for 8h, add 20 µl of the CCK-8 solution to each well of the plate. Then incubate the plate for 3 hours. Finally, measure the absorbance at 450 nm using a microplate reader.

Hoechst 33342 and PI fluorescent microscopy

Hoechst 33342 and PI fluorescent microscopy analyses were performed to distinguish cell death. According to the protocols, the treated cells were harvested and resuspended in 1 ml RPMI-1640 medium, Hoechst 33342 were added and further incubation for 10 min in dark. Then the cells were resuspended again in 1 ml PBS, PI (5 µg/ml) was added. Images were obtained using a fluorescent microscope excited at 350 and 530 nm for Hoechst 33342 and PI respectively. The photographs were merged and analyzed by Act-2U software.

Flow cytometry analysis of cell apoptosis

Annexin V and PI flow cytometry was performed according to the manufacturer's instructions. Simply, a further conventional incubation for 12h after treatment, then the cells were harvested and washed twice with ice-cold PBS. The cells were resuspended at a density of 1x10⁶ cells/ml in 1X buffer, added with Annexin V-FITC (5 µl) and PI (5 µl). After incubation for 30min, the cells were detected with FACS Calibur and the data were analyzed by CellQuest software.

In vitro invasion and metastasis assay

Cells (3x10⁴) were resuspended in RPMI Medium 1640 without FBS and placed into uncoated or Matrigel-coated Transwell inserts containing 8-µm filters (Corning Transwell) in triplicate. The bottom wells contained RPMI Medium 1640 with 20% fetal bovine serum (FBS). After 24h, the cells on the upper surface of the filters were removed with a cotton swab. The filters were fixed and stained with a 0.1% crystal violet solution and photographed. The migrated cells were then counted.

Western blotting

Cells were harvested in RIPA lysis buffer(150mM NaCl, 10mM Tris, pH 7.5, 1%NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail). Protein concentration was determined using the BCA Protein Assay Kit, and protein samples were resolved on 10% SDS-Page gels, transferred to the PVDF membrane, blocked in 5% non-fat milk in PBS/Tween-20, and blotted with the antibodies for Bax, bcl-2. GAPDH was used as loading controls. All antibodies were used at a 1:5000 dilution.

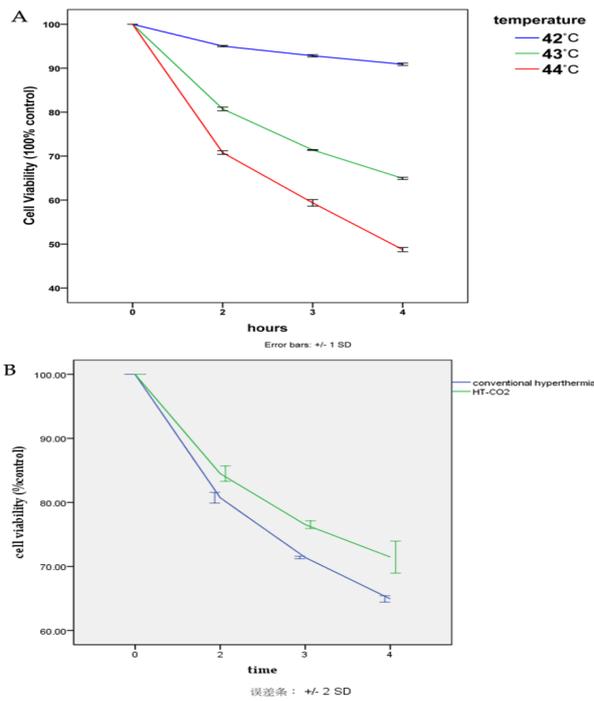


Figure 2. Cytotoxic Effect of Hyperthermic CO₂ Pneumoperitoneum (HT-CO₂) on Human Gastric Cancer AGS Cells

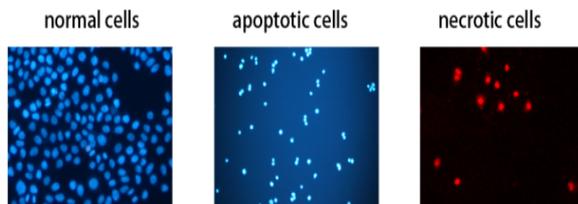


Figure 3. Morphological Analysis of HT-CO₂ Induced Cell Death (Hoechst 33342/PI fluorescent microscopy x200)

Statistical analysis

All the experiments were conducted at least for three times. All data were expressed as mean \pm SD. Statistical analysis were performed by using spss 13.0, t-test. A level of $P < 0.05$ was considered significant in all statistical tests.

Results

HT-CO₂ exhibited significant toxicity on human gastric cancer cells

HT-CO₂ induced obvious inhibition of cell viability on human gastric cancer cells. As shown in Figure 2A, HT-CO₂ (43°C, 2h) led to a significantly decrease in viable cells ($P < 0.05$), indicating a intensive anti-tumor effect against human gastric cancer cells. Furthermore, as seen in Figure 2B, the results testified that the anti-tumor effect of HT-CO₂ was better than conventional hyperthermia at the same temperature and time (43°C, 2h, 80.73% \pm 0.41%VS85.8% \pm 1.89%, $P < 0.05$). These results evidenced that HT-CO₂ truly had a conspicuous anti-tumor effect on human gastric cancer cells.

HT-CO₂ induced apoptosis in human gastric cancer AGS cells

To characterized the cell death induced by HT-CO₂, we inspected the nuclear morphology of dying cells. After

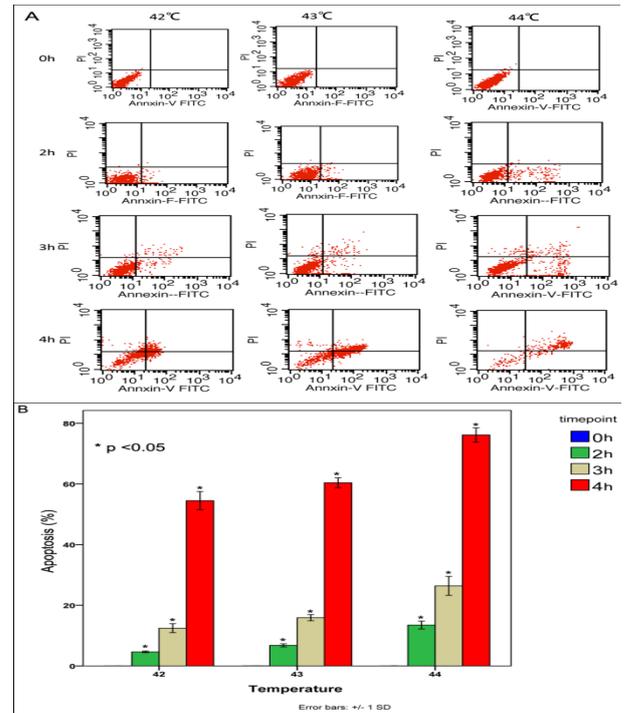


Figure 4. Analysis of Annexin V and PI Flow Cytometry Indicated Significant Temperature and Time Dependent Induction of Apoptosis in Human Gastric Cancer Cells

treated by HT-CO₂, the cells were dealt with Hoechst 33342/PI fluorescent microscopy in order to distinguish cell apoptosis from necrosis. The results indicated that apoptosis was the major form of cell death (Figure 3). We could see that AGS cells exposed to HT-CO₂ (43°C, 2h) showing specific changes in the cell including nuclear condensation and fragmentation (deep blue stained); with the increase of processing temperature and time (HT-CO₂ 44°C for 3h), the form of cell death was mainly necrosis (red stained). Furthermore, we also quantified the degree of apoptosis using Annexin-V/PI flow cytometry. As shown by Figure 4, when the cells were treated at 42°C for 2h, the early phase apoptosis was the main form of apoptosis, however, with the increase in processing temperature and time, the proportion of late phase apoptosis gradually increased. We could draw the following that HT-CO₂ induced cell apoptosis in a temperature- and time- dependent manner.

HT-CO₂ exhibited notable inhibition on migratory and invasive capacity of human gastric cancer AGS cells

We compared the effect of and HT-CO₂ and conventional hyperthermia on cell invasion and metastasis at the same temperature and time. After treated by HT-CO₂ and conventional hyperthermia at 43°C for 3 hours, an in vitro invasion assay indicated that a significant decrease in the numbers of cells migrating through the collagen membrane relative to the conventional incubation group, but the HT-CO₂ exhibited a better suppression effect than the conventional hyperthermia (~8.4 cells vs. ~3.3 cells per HPF; $P < 0.05$; Figure 5a). The same phenomenon was also observed in the metastasis assay. The cells invading through the Matrigel-coated membrane in the HT-CO₂ group was less relative to the conventional hyperthermia

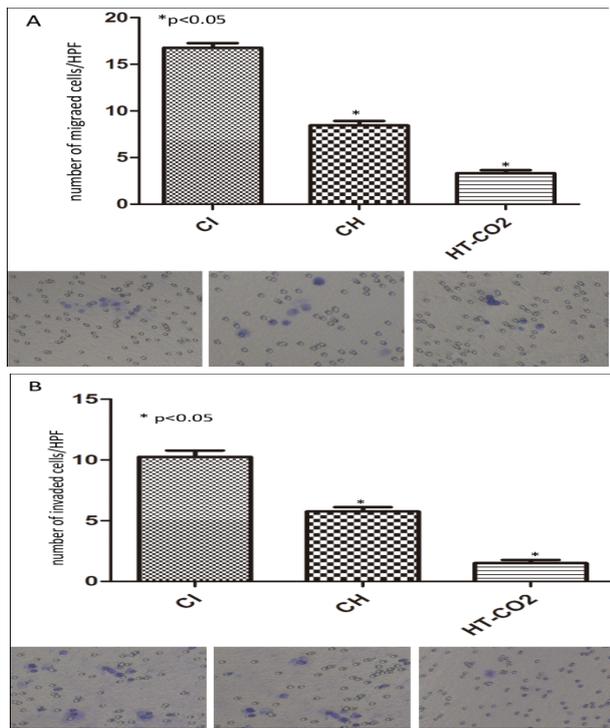


Figure 5. Effect of Conventional Hyperthermia and HT-CO₂ on Migratory and Invasive Capacity of Gastric Cancer AGS Cells

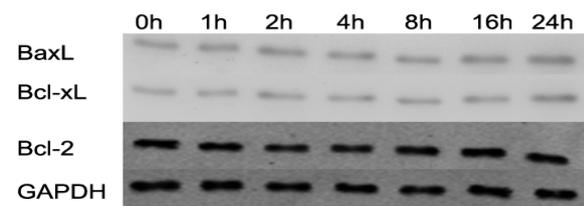


Figure 6. HT-CO₂ Induced Apoptosis of AGS Cells Through Bax-associated Mitochondrial Signal Pathway

group (~5.7 cells vs. ~1.5 cells per HPF; $P < 0.05$; Figure 5b).

HT-CO₂ induced apoptosis through Bax-associated mitochondrial signal pathway

Several gene products were known to play pivotal roles in modulating the apoptotic process. The imbalance of the expression anti- and pro-apoptotic protein was one of the major mechanisms in deciding the ultimate fate of cells. We inspected the expression of one pro-apoptotic protein, Bax, which form a large channel, allowing the release of cytochrome c; this process can be prevented by Bcl-2 or Bcl-xL (PAN et al., 2005). Western blot showed that the expression of Bax was upregulated while no change in the expression level of Bcl-2 and Bcl-xL. The results implied that Bax associated mitochondrial signal pathway played a critical role in HT-CO₂ induced apoptosis.

Discussion

Gastric cancer with peritoneal carcinomatosis has a poor prognosis and quality of life. In the past two decades, many compelling functional studies indicated that the programmed cell death by apoptosis could served

as a natural barrier to cancer development (Scott et al., 2004; Adams et al., 2007). Adams and Cory reported that apoptosis was attenuated in those tumors that succeeded in progressing to states of high grade malignancy and resistancy (Adams et al., 2007). The apoptotic program involved both upstream regulators and downstream effectors. The Bcl-2 family of proteins, including pro-apoptotic Bad, Bax and anti-apoptotic Bcl-2, Bcl-xL, are key regulators of mitochondrial mediated apoptosis (Green et al., 2004). The Bcl-2, along with its subfamily members (Bcl-XL, Bcl-w, Mcl-1, A1) were inhibitors of apoptosis, acting in largely by binding to and thereby suppressing the other two proapoptotic proteins (Bax and Bak), the latter located in the mitochondrial outer membrane. Bax and Bak could disrupt the integrity of the outer mitochondrial membrane, result in the release of proapoptotic signaling proteins: cytochrome c. The released cytochrome c activates, in turn, a cascade of caspases that act via their proteolytic to induce the multiple cellular changes associated with the apoptotic program (Willis et al., 2005; Adams et al., 2007).

Many recent studies have indicated that hyperthermia act through the induction of apoptosis to prevent tumor progression. A Goto et al. examined the role of the p53 gene in hyperthermia-induced apoptosis using three human gastric carcinoma cell lines, MK28 (carrying mutated type p53 gene), MKN-74 (wild-type), and KATO-III (complete deletion), the results indicated that hyperthermia triggered apoptosis could occur both in a p53 gene-dependent and -independent manner (A Goto et al., 1999). Satoshi Kokura reported that hyperthermia alone (at 43°C) killed cancer cells independent of the NF-κB transcription pathway (Kokura et al., 2003). Based on the newly-devised in vitro study model, we clarified the molecular mechanisms by which HT-CO₂ triggered human gastric carcinoma AGS cell undergoing apoptosis. In summary, our results clearly demonstrated that HT-CO₂ played a significant role in anticancer on human gastric carcinoma AGS cells in a temperature- and time-dependent manner. The cytotoxicity was associated with hyperthermia, cellular acidosis and hypoxia by the HT-CO₂. HT-CO₂ has several potential benefits; first, HT-CO₂ may induce apoptosis, denature proteins and impair DNA repair. Second, HT-CO₂ allows for greater drug accumulations within tumor nodules (Gill et al., 2011). HT-CO₂ could also result in extracellular acidification. It has, however, been shown that cellular pH is crucial for biological functions such as cell proliferation, invasion and metastasis, drug resistance and apoptosis.

On the other hand, hypoxia induced by the HT-CO₂ could also lead to intracellular and extracellular acidosis. Cellular acidosis has been a trigger in the early phase of apoptosis and leads to activation of endonucleases including DNA fragmentation (Izumi et al., 2003). Satoshi Kokura et al. reported that hyperthermia alone was significantly cytotoxic at 43 °C on MNK45 gastric cancer cells (Kokura et al., 2003). Our studies have provided convincing evidence that the AGS gastric cancer cells exposed to the at 42-44 °C HT-CO₂ for approximately 2-4 hours exhibited conspicuous tumor suppression, more importantly, the cytotoxic effect of was significantly

more than that of hyperthermia alone, which was due to the multiple roles of hyperthermia, cellular acidosis and hypoxia.

Neoplastic epithelial cells coexist in carcinomas with a biologically complex stroma composed by various types of stroma cells and extracellular matrix (ECM), both of which lead to the complexity of the tumor microenvironment (Mina et al., 2001; Margareta et al., 2004). Sooner or later during the development of most types of human cancer, the primary tumor cells invade adjacent tissues, and hence travel to distant sites where they may succeed in founding new colonies. The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade (Talmadge et al., 2010): the primary tumor cells invade adjacent tissue, enter the systemic circulation (intravasate), translocate through the vasculature, arrest in distant capillaries, extravasate into the surrounding tissue parenchyma, and finally proliferate from microscopic growth rate (micrometastases) into macroscopic secondary tumors (Isaiah et al., 2003). In 2000, we had little knowledge of the mechanisms underlying invasion and metastasis. However, the important role of cell-ECM interactions in the invasion and metastasis has been established (Guo et al., 2004; Jay et al., 2010). The tumor cell integrin binds to ECM ligands activating a form of intracellular signaling promoting cell invasion and metastasis; this type of adhesion molecules dependent intracellular signal transduction was generally called "outside-in signaling" (Giancotti et al., 1999). On the other hand, matrix metalloproteinases (MMPs) were a family of zinc-dependent neutral endopeptidases that had a crucial role in the degradation of extracellular matrix, which were crucial for malignant tumor growth, invasion, and metastasis (Zucker et al., 2004). Ozgur Kemik et al. investigated MMP-1 level in gastric cancer patients and compared them with a control group. The results indicated that higher serum MMP-1 level was observed in patients than in controls. Serum MMP-1 level was positively associated with morphological appearance, tumor size, depth of wall invasion, lymph node metastasis, liver metastasis, peritoneal invasion, and pathological stage (Kemik et al., 2011). In our study, we demonstrated that HT-CO₂ as a novel therapy could inhibit cell invasion and metastasis. Hyperthermia can alter the expression level of matrix metalloproteinase (MMP) and adhesion molecules to inhibit tumor invasion and metastasis. Takashi Sato et al. reported that heat shock preferentially suppresses MMP-1 production, and thereby inhibits proMMP-2 activation in HT-1080 cells, which eventually results in the inhibition of tumor invasion and metastasis in vitro (Sato et al., 1999). Hideharu Fukao et al. reported that hyperthermia would inhibit the invasion and metastasis activity of carcinoma cells through downregulation of the expression level of u-PAR (Hideharu et al., 2000). This study demonstrated that HT-CO₂ was able to inhibit AGS cell invasion and metastasis. However, further research of the molecular mechanism of this effect is urgently needed.

In summary, our results clearly demonstrate that the HT-CO₂ triggered apoptosis in a temperature- and time-dependent manner in AGS cells. More importantly,

the HT-CO₂ could also inhibit AGS cell invasion and metastasis. Analyses of expression of the Bcl-2 family proteins suggested that apoptosis induced by HT-CO₂ in AGS cells was mainly associated with Bax induced mitochondrial pathway.

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