

Thermotherapy enhances oxaliplatin-induced cytotoxicity in human colon carcinoma cells

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Abstract

AIM: To observe the synergistic effects of hyperthermia in oxaliplatin-induced cytotoxicity in human colon adenocarcinoma Lovo cells.

METHODS: The human colon adenocarcinoma cell line Lovo was obtained from Sun Yat-Sen University. Cells were sealed with parafilm and placed in a circulating water bath, and was maintained within 0.01 °C of the desired temperature (37 °C, 39 °C, 41 °C, 43 °C and 45 °C). Thermal therapy was given alone to the nega-

tive control group while oxaliplatin was administered to the treatment group at doses of 12.5 µg/mL and 50 µg/mL. Identification of morphological changes, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, flow cytometry and Western blotting were used to investigate the effect of thermochemotherapy on human colon adenocarcinoma Lovo cells, including changes in the signal pathway related to apoptosis.

RESULTS: A temperature-dependent inhibition of cell growth was observed after oxaliplatin exposure, while a synergistic interaction was detected preferentially with sequential combination. Thermochemotherapy changed the morphology of Lovo cells, increased the inhibition rate of the Lovo cells ($P < 0.05$) and enhanced cellular population in the G₀/G₁ phase ($16.7\% \pm 4.8\%$ in phase S plus $3.7\% \pm 2.4\%$ in phase G₂/M, $P < 0.05$). Thermochemotherapy increased apoptosis through upregulating *p53*, *Bax* and downregulating *Bcl-2*. Protein levels were elevated in *p53*, *Bax*/*Bcl-2* in thermochemotherapy group as compared with the control group ($P < 0.05$).

CONCLUSION: Thermochemotherapy may play an important role in apoptosis *via* the activation of *p53*, *Bax* and the repression of *Bcl-2* in Lovo cells.

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Key words: Colorectal cancer; Oxaliplatin; Thermochemotherapy; Mitochondrial apoptotic pathway

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer worldwide^[1,2]. Over the past decades, the incidence of CRC has been increasing in China, with an increased mortality due to early metastases^[3]. Despite surgical resection, more than 40% of the CRC patients die of the metastases ultimately^[4-6]. The poor prognosis is associated with difficulties in early diagnosis at a curable stage. Therefore, the urgent need to develop new therapeutic strategies in order to significantly improve the prognosis of the metastatic CRC patients is not overstated.

Oxaliplatin, a new third-generation platinum coordination complex of the 1,2-diaminocyclohexane family, has shown promising activity for CRC^[7-9]. Its spectrum of antitumor activities in tumor models differs from that of cisplatin and carboplatin. Oxaliplatin is active in a broad range of cancer cell lines and does not produce cross-resistance of cisplatin and carboplatin. Its side effects are also distinct from other platinum drugs—it induces no renal or hepatic toxicity but causes both a reversible acute, cold-related dysesthesia and a dose-limiting cumulative peripheral sensory neuropathy that usually rapidly regresses after treatment withdrawal^[10].

It is well known that hyperthermia is a strong factor that increases tumor sensitivity to chemotherapy^[11,12]. Cells in the DNA synthetic phase of the cell cycle are relatively resistant to chemotherapy, but are especially sensitive to hyperthermia. Thus, a combined treatment may, in some circumstances, be an advantage^[13]. Previous data have shown that the cytotoxicity and anti-tumor effect of cisplatin are greatly enhanced at elevated temperatures^[14]. It is also reported that the effect of oxaliplatin on colon cells is thermally enhanced at 42 °C when compared with 37 °C^[15].

A key mechanism associated with cancer cell growth is the control of apoptosis. Apoptosis is defined as programmed cell death, which occurs in response to disruption of normal homeostatic mechanisms. It is a critical parameter in tumor surveillance of abnormal cells^[16]. Induction of apoptosis of cancer cells is considered a potentially new treatment for colon cancer^[17].

However, whether the antitumor effect of oxaliplatin in colon cancer has a linear relationship with temperature is not known. Therefore, we explored the effect of thermal treatment in colon cancer cells and investigated the optimal temperature for the inhibitory effect of oxaliplatin in colon cancer cell lines.

MATERIALS AND METHODS

Major reagents

Newborn calf serum and Dulbecco's modified Eagle's medium (DMEM, low glucose) were purchased from GIBCO Corporation, penicillin/streptomycin was from Gibco-BRL (Germany), while 0.25% trypsin digest and trizol reagent were purchased from Invitrogen Corporation. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Corporation. Primary antibodies (applied for 1 h at room temperature or overnight at 4 °C) were: anti-Bcl-2, anti-Bax and anti-p53 monoclonal antibody all purchased from Santa Cruz Biotechnology (Heidelberg, Germany), goat anti-rabbit IgG (secondary) and prestained protein molecular weight marker were purchased from Cell Signaling Technology (United States).

Cell culture

The human colon adenocarcinoma cell line Lovo was obtained from the Cell Bank of Sun Yat-Sen University. All cell culture operations were carried out in a sterile class II biological safety cabinet (Thermo Fisher, United States). The Lovo cell lines were cultured in DMEM containing 10% fetal bovine serum (GIBCO Corporation, Carlsbad, CA, United States), 50 U/mL of penicillin and 50 mg/mL of streptomycin (Invitrogen, Carlsbad, California, United States). The cells were grown in 25-mL and 75-mL flasks (Corning, New York, United States) in a humidified incubator at 37 °C with 5% CO₂ atmosphere (Thermo Fisher, United States). Cells were stained with Trypan blue (Sigma-Aldrich, St Louis, Missouri, United States) and then counted on a hemacytometer.

Hyperthermia and oxaliplatin treatment

Cells cultured in 60-mm dishes were sealed with parafilm and placed in a circulating water bath (Yiheng Corporation, Shanghai, China), and was maintained within 0.01 °C of the desired temperatures (37 °C, 39 °C, 41 °C, 43 °C, 45 °C). Thermal therapy was given alone to the control group while oxaliplatin was administered to the treatment group at doses of 12.5 µg/mL and 50 µg/mL. The duration of thermal therapy and chemotherapy was 1 h, and the cells were cultured in a humidified incubator at 37 °C with 5% CO₂ atmosphere for 24 h.

Cellular morphological evaluation

The cells were observed under inverted phase contrast microscope (Olympus, Japan) after completion of thermal therapy or thermochemotherapy.

MTT assay

Measurement of cell growth inhibition by MTT assay was described previously^[18]. Lovo cells were seeded in 96-well plates (5 × 10³ cells/well) and were treated with 12.5 µg/mL or 50 µg/mL oxaliplatin at 37 °C, 39 °C, 41 °C, 43 °C or

45 °C. Negative control wells contained only CRC cells but not oxaliplatin. The experiment was repeated 3 times. Untreated and treated cells were cultured at 37 °C with 5% CO₂ for 24 h. MTT was added to 50 µL of cell suspension for 4 h. After supernatant was removed, DMSO (150 µL) was added to each well and mixed at a low speed for 10 min to fully dissolve the blue crystals. Absorbance was measured at 570 nm (A_{570}) and the percentage of growth inhibition of Lovo cells was calculated at each time point and for each concentration of oxaliplatin according to the following formula: % cell survival = (Lovo oxaliplatin group - Lovo blank)/(Lovo negative - Lovo blank) × 100% and % cell growth inhibition = 1 - % cell survival.

The fraction for each concentration of oxaliplatin was calculated. Model parameters included the concentration to inhibit 50% of cell growth (IC₅₀). At least three independent experiments were carried out to test the relationship between temperature of drug exposure and the concentration of oxaliplatin.

Flow cytometric analysis

Cells were plated at 1×10^6 cells/well in 6-well plates, incubated for 24 h, and then treated with different concentrations of oxaliplatin for 1 h. Trypsinized cells were washed with PBS and fixed in 70% ethanol. After fixation, the cells were incubated for 30 min with 200 mg/mL of RNase A and stained with 25 µg/mL propidium iodide (PI). The stained cells were analyzed using a flow cytometry cell sorter (Becton Dickinson, NJ, United States). Samples were analyzed using a FACScan flow cytometer (Becton Dickinson) according to the manufacturer's protocol. Experiments were performed in triplicate.

Apoptosis was measured according to the manufacturer's instructions, using an annexin V-FITC kit (BD Biosciences, San Jose, CA, United States). The cells were collected after drug delivery, washed twice with PBS and then centrifuged. The cell pellet was resuspended in ice-cold binding buffer. The annexin V-FITC and PI solutions were added to the cell suspension and mixed gently. The samples were then incubated for 15 min in the dark before flow cytometric analysis^[19]. The analysis of the apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson, NJ, United States).

Western blotting analysis

Lovo cells (cultured in 6-well plate at 1.5×10^5 cells/well) were treated with 12.5 µg/mL or 50 µg/mL oxaliplatin at 37 °C, 39 °C, 41 °C, 43 °C or 45 °C and total proteins were extracted. Protein samples were separated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, United States). The membrane was blocked overnight at 4 °C in TBSTween 20 (TBST) buffer containing 5% skimmed milk powder. The membrane was washed with TBST (3 × 8 min). Membranes were then incubated overnight at 4 °C in primary antibody (125 µL/cm³; diluted 1:1000) with gentle shaking. The membranes were washed with TBST (3 × 8 min) and incubated for 1 h at room temperature

in horse radish peroxidase (HRP)-conjugated secondary antibody (125 µL/cm³; diluted 1:2500). The membranes were washed with TBST (3 × 8 min) and protein signals were detected by chemiluminescence kit (Cell signaling Technology, United States). Primary antibodies (applied for 1 h at room temperature, or overnight at 4 °C) were: anti-Bcl-2, anti-Bax and anti-p53 monoclonal antibody all purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Statistical analysis

Normally distributed continuous variables were compared by one-way analysis of variance (ANOVA). Statistical analyses were performed using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL). When a significant difference between groups was apparent, multiple comparisons of means were performed using the Holm-Bonferroni procedure with type-I error adjustment. Data were presented as means ± SD. All statistical assessments were two-sided and evaluated at the 0.05 level of significant difference.

RESULTS

Cellular morphology

Lovo cells were treated with oxaliplatin (12.5 µg/mL or 50 µg/mL) at different temperatures for 1 h and then cultured under normal conditions for 24 h. There were disparities after treatment between the control group and the thermochemotherapy group (Figure 1). Thermochemotherapy alone caused the inhibition of cell growth by inducing cell apoptosis and cell cycle arrest. However, thermochemotherapy caused a greater decrease of Lovo cells as compared with the control group, especially in the 50 µg/mL group. The effect of thermochemotherapy on tumor growth was observed as early as 24 h after treatment.

Thermotherapy enhances oxaliplatin-induced inhibition of cytotoxicity

First, we used the MTT assay to detect the effect of temperature on Lovo cell proliferation. With increasing temperature, the inhibition rate of cell growth became higher and higher. The cytotoxicity of Lovo cells exposed to 50 µg/mL oxaliplatin for 1 h was higher than those exposed to 12.5 µg/mL oxaliplatin at the same temperature ($P < 0.05$). And 43 °C was the optimal temperature to inhibit cell proliferation when the cells were exposed to 50 µg/mL oxaliplatin ($P < 0.05$, Figure 2).

Thermotherapy enhances oxaliplatin-induced cell cycle arrest and apoptosis

To elucidate the mechanism of action of thermotherapy and oxaliplatin, we used flow cytometry to determine cell cycle distribution and apoptosis in Lovo cells exposed to different temperatures.

A significant increase in the number of G₀/G₁ phase cells and a decrease in the number of S and G₂/M phase

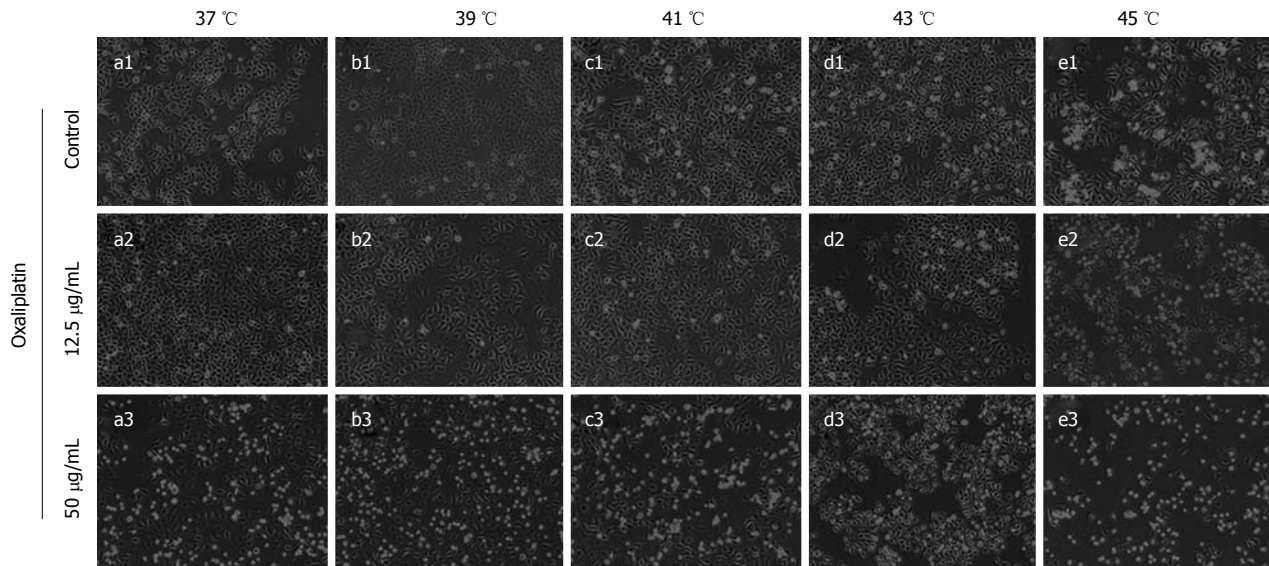


Figure 1 Effect of thermochemotherapy on human colon carcinoma Lovo cells. Cells were treated with oxaliplatin (12.5 µg/mL and 50 µg/mL) at different temperatures (37 °C, 39 °C, 41 °C, 43 °C or 45 °C) for 1 h and then cultured under normal conditions for 24 h. The cells were observed under inverted phase contrast microscope (Olympus, Japan) after completion of thermal therapy or thermochemotherapy.

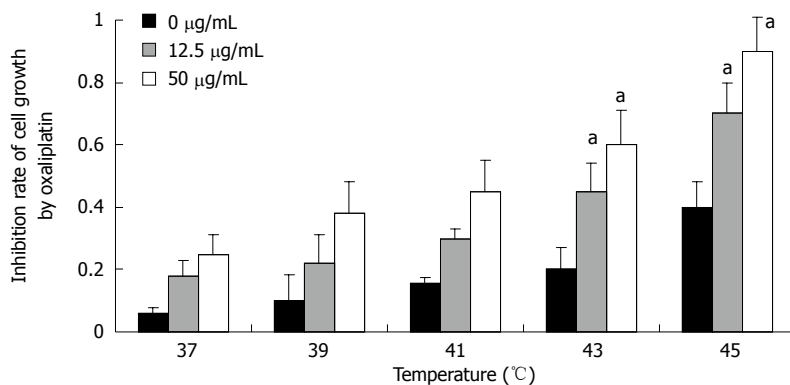


Figure 2 Effect of temperature on the proliferation of Lovo cells by MTT assay. Lovo cells were treated with oxaliplatin (12.5 µg/mL and 50 µg/mL) at 37 °C, 39 °C, 41 °C, 43 °C or 45 °C. The control group only used thermal therapy but not oxaliplatin. Statistically significant differences were observed between the two groups. Lovo cell viability was determined by MTT assay. The white bars are the results of the oxaliplatin treatment for 1 h at 37 °C, 39 °C, 41 °C, 43 °C, and 45 °C followed by 24 h recovery. The results of three independent experiments are reported as mean ± SD. ^a*P* < 0.05 vs 43 °C or 45 °C vs 37 °C.

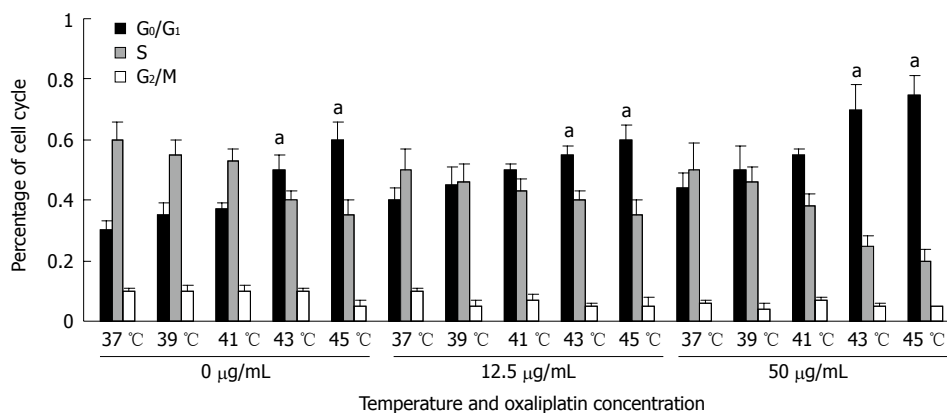


Figure 3 Effect of temperature on cell cycle and apoptosis detected by flow cytometry. Lovo cells were treated with oxaliplatin (12.5 µg/mL and 50 µg/mL) at 37 °C, 39 °C, 41 °C, 43 °C or 45 °C for 1 h. The different bars represent the percentage of Lovo cells at different phases of the cell cycle at different temperature points. ^a*P* < 0.05 vs 43 °C or 45 °C vs 37 °C.

cells after 1 h of oxaliplatin treatment were observed. There was a linear relationship between the cell cycle

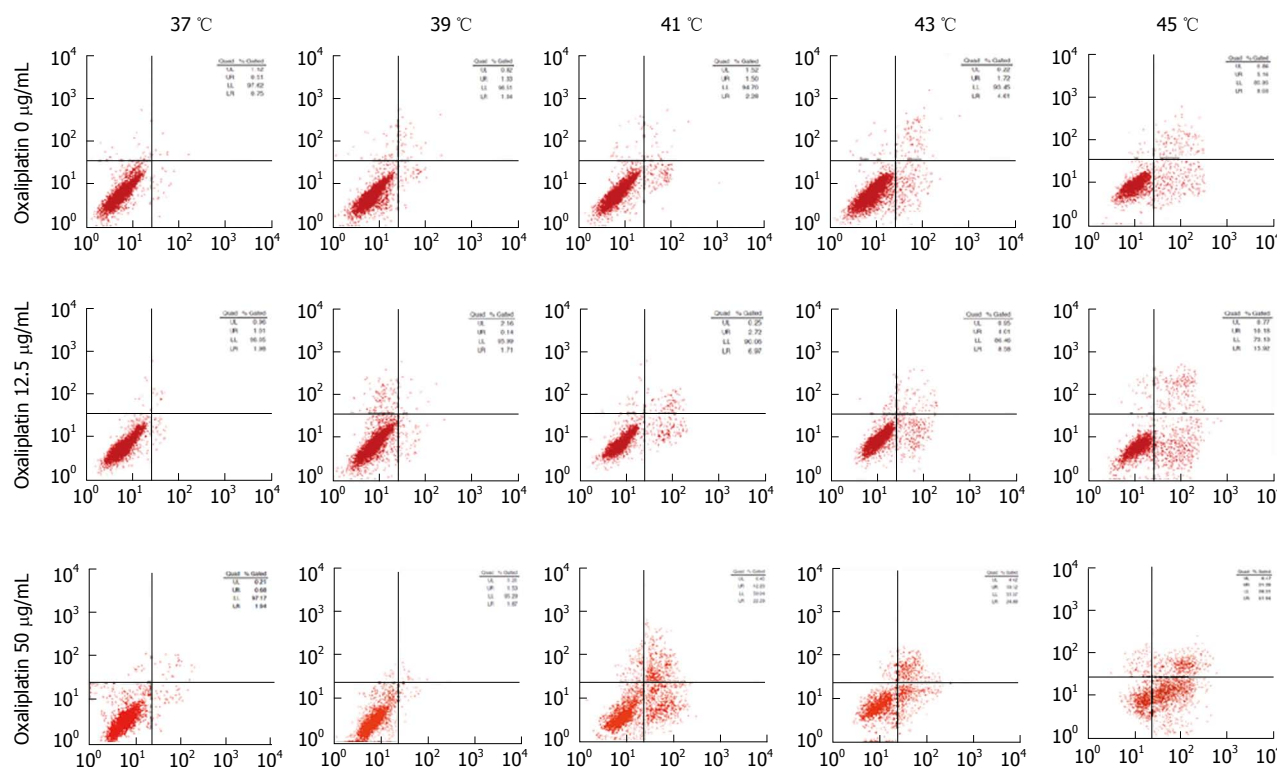


Figure 4 Measurement of Lovo cell apoptosis using apoptosis detection kit. Data are presented as dot plots in which the vertical axis represents propidium iodide (PI)-positive cells and the horizontal axis annexin V-positive cells. The upper left quadrant region contains necrotic (PI-positive) cells, the upper right region contains the late stage of apoptotic and necrotic (PI- and annexin V-positive) cells, the lower left region contains viable non-apoptotic (PI- and annexin-V-negative) cells, and the lower right region contains early apoptotic (PI-unstained and annexin-V-positive) cells.

and the temperature ($P < 0.05$). The proliferation and proportions of cells in different phases of the cell cycle were analyzed at 24 h by the incorporation of PI. DNA histogram analysis revealed that thermal therapy induced a temperature-dependent increase in the number of cells within the G_0/G_1 phase. This increase was accompanied by a decrease in the percentage of proliferating cells ($16.7\% \pm 4.8\%$ in phase S and $3.7\% \pm 2.4\%$ in phase G_2/M) ($P < 0.05$, Figure 3). Accumulation of $12.5 \mu\text{g/mL}$ oxalipatin-treated cells at any phase was less remarkable than that of the $50 \mu\text{g/mL}$ -treated cells ($P < 0.05$).

We also used PI staining to show that thermotherapy induced apoptosis of Lovo cells in a temperature-dependent manner (Figure 4).

Hyperthermia enhances oxalipatin induced-regulation of p53 and Bax/Bcl-2

It is well known that reduction of intra-cellular apoptotic molecules, such as p53 and Bax/Bcl-2, sensitizes Lovo cells to thermotherapy. We therefore investigated whether changes in the amounts of apoptotic proteins were associated with the promotion of hyperthermia and oxalipatin.

p53 stimulated the mitochondrial apoptotic pathway, thus enabling direct protein interaction or inhibition of the Bcl-2 protein family. p53 can also induce the pro-apoptotic Bcl-2 proteins by transcribing or inhibiting the transcription of anti-apoptotic Bcl-2 proteins. We examined the effect of thermal therapy on the expression of the Bcl-2 protein group.

The results showed a thermal-dependent increase in Bax expression over temperature and a concomitant decrease in the expression of Bcl-2. The maximal levels of Bax reached a peak at 43°C . As for Bcl-2, there was a marked decrease when compared 43°C and 45°C with 37°C . These levels later increased somewhat but always remained lower than that in the control group ($P < 0.05$). Both Bax activation and Bcl-2 inhibition were required for the release of mitochondrial apoptotic factors and the activation of the intrinsic apoptotic route.

Finally, we detected the possible signal pathway involved in the effects of oxalipatin on Lovo cells. There was an increase in the expression of p53 and Bax protein in cells treated with oxalipatin for 1 h. Compared with the cells of the control group, a gradual decrease in Bcl-2 levels was found at an increasing temperature, with the most significant reduction at 43°C (Figure 5).

DISCUSSION

Thermotherapy combined with immediate intraperitoneal delivery of adjuvant anti-cancer drugs is a novel cancer treatment strategy to improve prognosis and prolong the overall survival of the patients with advanced CRC. The aim of this study was to assess the role of hyperthermia in oxalipatin-induced cytotoxicity, to find an appropriate temperature using *in vitro* studies in maximizing anti-tumor activities and discuss the possible mechanism of thermochemotherapy. Preclinical studies have suggested

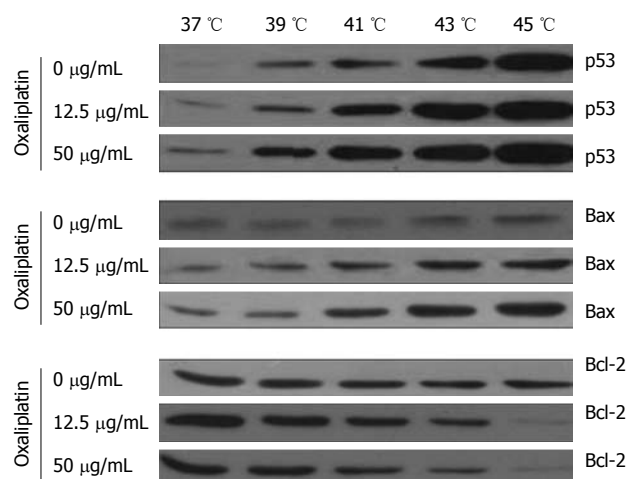


Figure 5 Effect of thermal therapy on p53, Bax and Bcl-2 detected by Western blotting. Lovo cells were treated with 12.5 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ oxaliplatin for 1 h at 37 $^{\circ}\text{C}$, 39 $^{\circ}\text{C}$, 41 $^{\circ}\text{C}$, 43 $^{\circ}\text{C}$ or 45 $^{\circ}\text{C}$. Cells were harvested, and total proteins were extracted and immunoblotted for p53, Bax and Bcl-2. The values represent means \pm SD of at least three separate experiments. Beta-actin was used as loading control (data not shown).

that oxaliplatin may offer therapeutic advantages in a variety of malignancies with either intrinsic or acquired cisplatin resistance^[20]. The primary mechanism of oxaliplatin has been shown to be mediated by the formation of intrastrand DNA cross-links^[21]. Rietbroek *et al.*^[22] reported that thermotherapy at 43 $^{\circ}\text{C}$ enhanced the formation of DNA cross-links and concluded that a large portion of enhanced cytotoxicity may be attributed to the increased cross-links. Other factors such as an increased drug uptake at the elevated temperature are also suggested^[23]. Evidence that oxaliplatin exerts a specific anti-tumor effect strongly suggests that oxaliplatin may be a promising new compound for the treatment of gastrointestinal tumors^[24-26].

The *in vitro* exposure of human Lovo cells at clinical concentration of oxaliplatin with thermotherapy exerted a strong anti-proliferative effect and induced apoptosis of Lovo cells in this study. Our results highlight the potential clinical value of thermochemotherapy in the treatment of colon cancer. The exposure to 43 $^{\circ}\text{C}$ or above augmented the cytotoxicity of the oxaliplatin-treated Lovo cells in the thermochemotherapy group in a temperature-dependent fashion. This effect was observed by the MTT assay which demonstrated a linear increase in cytotoxicity with thermotherapy. After exposure to 43 $^{\circ}\text{C}$, the activity of oxaliplatin markedly and rapidly increased, indicating its potential inhibition ability in Lovo cells. Consistently, previous studies have demonstrated that oxaliplatin treatment with different concentrations (7.5-39.7 $\mu\text{g/mL}$) for 5-150 min can enhance cell death in various cell lines^[27,28].

The exact mechanisms of thermochemotherapy remain unclear. Exposure to high temperatures can alter the fluidity of cell membranes, inhibit protein synthesis, and destroy DNA synthesis enzymes. Regulation of the cell cycle and apoptosis is a major strategy to inhibit the progression of a number of cancers. A critical role of

p53, a sequence-specific DNA-binding protein, has been demonstrated to execute apoptosis. Either thermotherapy or oxaliplatin can cause cell cycle arrest at the G₀/G₁ phase and can induce apoptosis of human colon cancer cells.

Some studies have reported that p53 changes cell-cycle arrest and apoptosis induction by regulating the expression of different proteins such as p21, Bax and Bcl-2^[29,30]. The Bcl-2 gene product functions as an anti-apoptotic signal, suppressing apoptosis induced by chemotherapeutic drugs. The exact mechanism of Bcl-2 in preventing apoptosis is still not clear. Others have described p53 expression in response to genotoxicity. It has been proposed that p53 may be involved in the cellular response to DNA damage, producing arrest in the G₀/G₁ phase of the cell cycle to allow efficient repair of DNA before entry into S phase^[31,32]. For these reasons, we inferred that the effects of thermotherapy in Lovo cells were mediated partly by the expression of p53.

Our results showed that thermotherapy could induce apoptosis and cell cycle arrest in Lovo cell lines in a temperature-dependent manner. Thermochemotherapy significantly induced cell cycle of G₀/G₁ phase arrest by upregulating p53 and Bax expression and downregulating Bcl-2 in Lovo cells. We also detected the expression of Bcl-2 and Bax in Lovo cells to understand the impact of thermotherapy on the mitochondrial pathway of apoptosis. Down-regulation of Bcl-2 and up-regulation of p53 and Bax synthesis showed that apoptosis induced by thermochemotherapy may be mediated by the mitochondrial pathway^[33,34]. Our findings were consistent with other studies^[35,36] that thermotherapy caused G₀/G₁ cell cycle arrest and promoted apoptosis of Lovo cells by upregulating p53, Bax expression and downregulating Bcl-2^[37,38].

In conclusion, our findings indicate that oxaliplatin is a promising agent for the treatment of human Lovo cells. Thermotherapy exerted synergistic interaction with oxaliplatin especially at 43 $^{\circ}\text{C}$ or above, inhibiting the survival of Lovo cells *in vitro*. Apparently, the observed synergism between thermotherapy and oxaliplatin results in mutual completion and enhancement of anticancer activity that may be extrapolated to animal models of colon cancer and to clinical use. The results of the present study suggest that thermochemotherapy might be a useful future strategy for treating colon cancers.

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COMMENTS

Background

Oxaliplatin is reported to be active in a broad range of cancer cell lines and hyperthermia is a strong factor that increases tumor sensitivity to chemotherapy. However, whether the antitumor effect of oxaliplatin in colon cancers has a linear relationship with temperature and the optimal temperature for the inhibitory

effect of oxaliplatin in colon cancer cell lines remains elusive.

Research frontiers

Thermochemotherapy may play an important role in the inhibition of the Lovo cells and the optimal temperature is 43 °C. In addition, down-regulation of Bcl-2 and up-regulation of p53 and Bax may be essential in inducing apoptosis in human colon cancer cell lines.

Innovations and breakthroughs

Thermal therapy and oxaliplatin can inhibit colon cancer cells through inducing apoptosis and regulating the cell cycle. Suppression of Bcl-2 and up-regulation of p53 and Bax might contribute to the regulation of human Lovo cell lines.

Applications

This study may help clarify the mechanism of thermochemotherapy for colon cancer and choose the appropriate therapeutic strategy in clinical practice.

Terminology

Thermochemotherapy is the combination of thermal therapy and chemotherapy.

Peer review

This is a study using a single cell line. The experiments are straightforward and the data are short and succinct.

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