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Thermochimica Acta 397 (2003) 87–95

thermochimica acta

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Microcalorimetric studies of the synergistic effects of copper-1,10-phenanthroline combined with hyperthermia on a liver hepatoma cell line Bel-7402

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Received 10 December 2001; received in revised form 10 April 2002; accepted 21 May 2002

Abstract

The effects of copper-1,10-phenanthroline combined with hyperthermia on human liver hepatoma cell line, Bel-7402 were studied. The effect was evaluated by the mean thermal power of the cells and total energy *Q* produced during the measurement period (35 h). It was found that the energy produced reduced after the treatment. Condensation of nuclear chromatin and apoptotic bodies can be observed from fluorescence microscope, which showed apoptosis occurs under the action of copper-1,10-phenanthroline combined with hyperthermia. The analysis by flow cytometry showed the proportion of apoptotic cells in the cell population increased. It indicates that the combination of hyperthermia and copper-1,10-phenanthroline has a synergetic effect on Bel-7402.

Keywords: Microcalorimetry; Hyperthermia; Copper-1,10-phenanthroline; Apoptosis

1. Introduction

The combination of different treatment modalities plays a major role in controlling primary tumor and microscopic metastatic disease [1]. Increased therapeutic activity from the use of radiation and different chemotherapeutic drugs has been demonstrated in experimental and clinical studies.

Phenanthroline is known to promote hydroxyl radical formation from molecular oxygen by redox-cycling, and therefore considered to be a suitable agent for the stimulation of ROS formation [2]. At the same time, copper-1,10-phenanthroline, $Cu^{II}(OP)_2$, is a chemical nuclease that nicks DNA [3] and can upregulate the DNA-binding activity of p53, which play an important role in regulating cell progression, cell survival and apoptosis [4].

For a long time now, biological studies have demonstrated that hyperthermia can cause lethal damage to both mammalian cells and human tumors [5], and can enhance the effect of radiation and some chemotherapeutic agents [6]. Hyperthermia seems due to the possible inhibition of the cellular repair of potentially lethal damage or to the inhibition of enzymes

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0040-6031/02/$ – see front matter
PII: S0040-6031(02)00331-3

responsible for DNA repair [1]. Studies show that hyperthermia can inhibit repair of both DNA double- and single-strand breaks and can inhibit DNA polymerase in a manner correlated to cell killing [7,8]. It was also reported that hyperthermia causes an increase in the tumor oxygenation status in a variety of experimental tumors [9,10].

The fact that $Cu^{II}(OP)_2$ and hyperthermia seem to have DNA as one of their targets may provide a rational for the use of combined treatment. But few studies are available on this combination.

Experimental and clinical studies have demonstrated that the combined treatment of hyperthermia and anti-tumor agents enhances tumor response [11,12]. The possibility of using $Cu^{II}(OP)_2$ as a chemotherapeutic agent has been investigated.

This paper investigated the effect of hyperthermia and $Cu^{II}(OP)_2$ on a liver hepatoma cell line (Bel-7402) by microcalorimetry, fluorescence microscopy and flow cytometry. These methods afford a set of way to qualify [13] and quantify [14] the clinical efficiency of drugs. Because many of these changes are very characteristic and appeared to be unique to apoptosis, they have become markers used to identify this mode of cell death biochemically by microscopy and cytometry. Furthermore, the proportion of apoptotic cells in the cell population can be detected by flow cytometry [15]. Heat production of cell line Bel-7402 under the action of $Cu^{II}(OP)_2$ combined with hyperthermia was studied by an LKB2277 Bioactivity Monitor. The effect was evaluated by the mean thermal power (P_{mean}) and the total energy (Q) produced during the measurement period (35 h). The morphology and DNA content of treated Bel-7402 indicated the cells died from apoptosis. The ratio of survival cells in the group at different treatment showed the combination of hyperthermia and $Cu^{II}(OP)_2$ enhanced their effect on cells, which can be a potential method to deal with cancer.

2. Experimental

2.1. Materials

RPMI-1640 and fetal calf serum were purchased from GIBCO Co, USA. Hoechst 33258, RNase A and PI was obtained from Sigma. Phenanthroline was obtained from Shanghai Chemical Factory (A.R.).

2.2. Cell culture

Bel-7402, human liver hepatoma cell line [16] was obtained from China Center for Type Culture Collection, Wuhan University.

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 5% CO_2 at 37.0 °C. The same medium was used for the calorimetric experiments.

2.3. Microcalorimetry assay

The cells were cultured in the flask and trypsinized to be count using a Thoma's hemocytometer and cell number was set to 2×10^5 cell ml^{-1} by adding the medium. Then 1 ml cell suspension were removed to a 3 ml glass ampoules. $Cu^{II}(OP)_2$ was added to the sample after the cells were cultured in ampoules for 24 h and then put into the microcalorimeter to monitor the growth of the cell. All the comparisons had been made after $Cu^{II}(OP)_2$ was added. The calorimetric measurement was carried out by using the ampoul method at 37.0 °C, which is corresponding to the physiological body temperature, and 40.0, 43.0 °C, i.e. hyperthermia, respectively.

A microcalorimeter, LKB 2277 Bioactivity Monitor (Thermometric AB, Sweden) was used to obtain the thermogenesis curves. For more details of the performance and construction of the instrument, see [17,18]. The cells were not stirred in the ampoule and the ampoules were electrically calibrated.

2.4. Chromatin morphology observation

The cells were cultured in the flasks and treated as same as the microcalorimetry assay. After treated for 35 h, cells were harvested and fixed in the mixture of methanol: acetic acid (3:1). Following centrifugation, the samples were stained by Hoechst 33258 and the morphology of nucleic was observed on BH-2 Fluorescence Microscope (Olympus Inc., Japan) [13].

2.5. Flow cytometry analysis

Untreated and treated cells were washed twice with PBS, and then fixed with 70% ethanol overnight at −20 °C. Fixed cells were washed with PBS, and

stained with 50 μg ml^{-1} PI contained 10 mg ml^{-1} RNase A. The DNA content of cells was analyzed on FACSort (Becton Dickinson) and the percentage of apoptotic cells determined using the CellQuest software program [14].

3. Results

3.1. Thermogenesis curves for cell line Bel-7402 under the action of $Cu^{II}(OP)_2$ at 37°C

The thermogenic curves for cell line at different concentrations of $Cu^{II}(OP)_2$ are shown in Fig. 1. From Fig. 1, we can see that at 37.0 °C the metabolism activity of cell line Bel-7402 was lower than that of control when the $Cu^{II}(OP)_2$ were added. The values of $J_{\Phi/N}$ (pW) per cell is 50 ± 10, which is almost the same as human white adipocytes [19]. The values of metabolic heat (Q) released during the experiment time were shown in Table 1.

The inhibitory ratio of $Cu^{II}(OP)_2$ was defined as: $I\% = (Q - Q_0)/Q \times 100$, in which Q is the total metabolic heat produced from the control cells during the experiment time and the Q_0 is the metabolic heat produced from the treated cells at 37.0 °C. The inhibitory ratio is also shown in Table 1. The value of inhibitory ratio versus the concentration of $Cu^{II}(OP)_2$ was shown in Fig. 2.

Table 1
The values of total metabolic heat released and the inhibitory ratio vs. different concentration of $Cu^{II}(OP)_2$

c (μg ml^{-1})	Q (J)	I (%)
0	1.45	
1	1.23	15.0
5	1.14	21.7
10	0.67	53.6
15	0.21	85.7
20	0.00	100

From Table 1 we can obtain the inhibitory ratio (I) versus the concentration of $Cu^{II}(OP)_2$ (c) obey the following equation:

$$I(\%) = 4.89c + 5.30, \quad (c: \text{ from } 0 \text{ to } 20\ \mu g\ ml^{-1}),$$

and $R = 0.987$

The half inhibitory concentration $c_{1/2}$ is defined as the concentration of $Cu^{II}(OP)_2$ that reduces the total metabolic heat to half the control value. In light of this definition, the half inhibitory concentration of $Cu^{II}(OP)_2$ is 9.1 μg ml^{-1}.

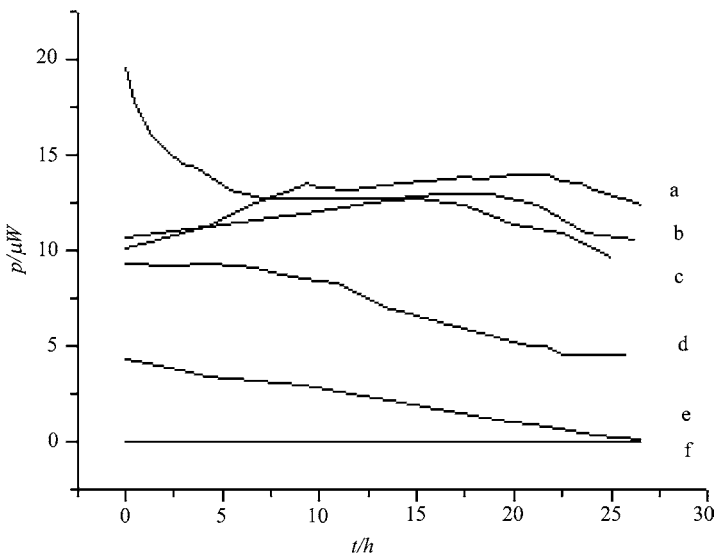

Fig. 1. The thermogenesis curves of cell line Bel-7402 at 37.0 °C with the concentration of $Cu^{II}(OP)_2$ at: 0 μg ml^{-1}; (b) 1 μg ml^{-1}; (c) 5 μg ml^{-1}; (d) 10 μg ml^{-1}; (e) 15 μg ml^{-1}; and (f) 20 μg ml^{-1}.

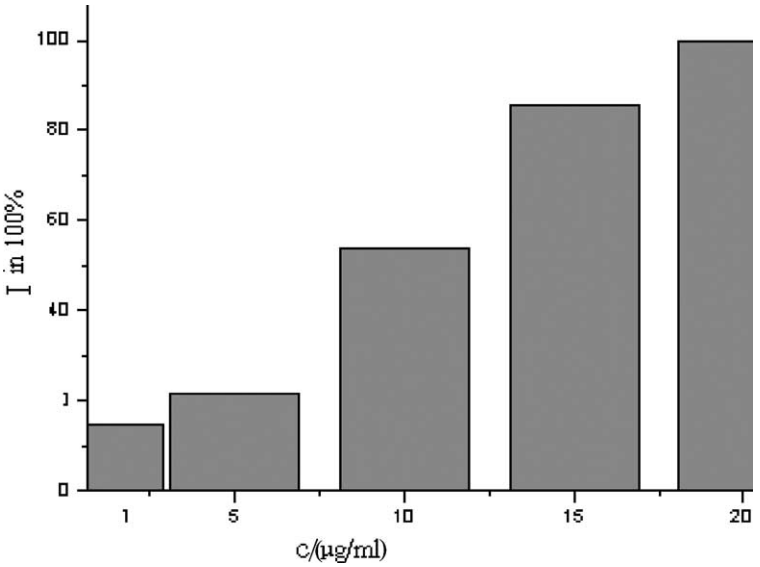

Fig. 2. The inhibitory ratio of different concentration of $Cu^{II}(OP)_2$. *I* is the inhibitory ratio, *c* is the concentration.

3.2. Thermogenesis curves for cell line Bel-7402 under the action of $Cu^{II}(OP)_2$ combined with hyperthermia

The thermogenesis curves for cell line Bel-7402 under the action of 10 μg ml^{-1} $Cu^{II}(OP)_2$ at 37.0, 40.0, 43.0 °C are shown, respectively in Fig. 3. We can obtain the total metabolic heat of the cell line Bel-7402 produced during the measurement time in presence of 10 μg ml^{-1} $Cu^{II}(OP)_2$. The values follow as:

$$Q(37.0\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 0.67\,\mathrm{J\,ml^{-1}}$$

$$Q(40.0\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 0.33\,\mathrm{J\,ml^{-1}}$$

$$Q(43.0\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 0.00\,\mathrm{J\,ml^{-1}}$$

The total energy (*Q*) produced during the measurement period is shown in Fig. 4. And the mean thermal powers per milliliter are:

$$P_{\mathrm{mean}}(37\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 7.2\,\mu\mathrm{W\,ml^{-1}}$$

$$P_{\mathrm{mean}}(40\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 3.44\,\mu\mathrm{W\,ml^{-1}}$$

$$P_{\mathrm{mean}}(43\,^{\circ}\mathrm{C} + \mathrm{Cu^{II}(OP)_2}) = 0.00\,\mu\mathrm{W\,ml^{-1}}$$

Analysis of the metabolic heat of the cells growing for 35 h is shown in Table 2, from which it was clear that hyperthermia can potentiate the cytotoxicity of copper-1,10-phenanthroline.

3.3. Chromatin morphology observation on fluorescence microscope

The photographs of Bel-7402 cells by means of a fluorescence microscope are shown in Fig. 5. A large number of apoptotic bodies were all detected in the cells treated with $Cu^{II}(OP)_2$ at 37.0, 40.0, or 43.0 °C. More importantly, the proportion of normal cells in the cell populations had significant reduction when treated by $Cu^{II}(OP)_2$ with the increasing of temperature. There are almost 90% normal cells in Bel-7402 treated with $Cu^{II}(OP)_2$ at 37.0 °C, and the ratio reduced to 70% when the treated cells cultured at 40.0 °C. When the temperature reached 43.0 °C, the normal cells had been hardly detected after treated. The results indicate the combination of hyperthermia and $Cu^{II}(OP)_2$ can induce apoptosis of Bel-7402.

Table 2
Comparison of metabolic heat released of cell line Bel-7402 cells

T (°C)	*Q* (J ml^{-1} (control))	*Q* (J ml^{-1} (+10 μg ml^{-1} $Cu^{II}(OP)_2$))	*I* (%)
37	1.45	0.67	53.64
40	2.26	0.33	85.39
43	0.22	0	100

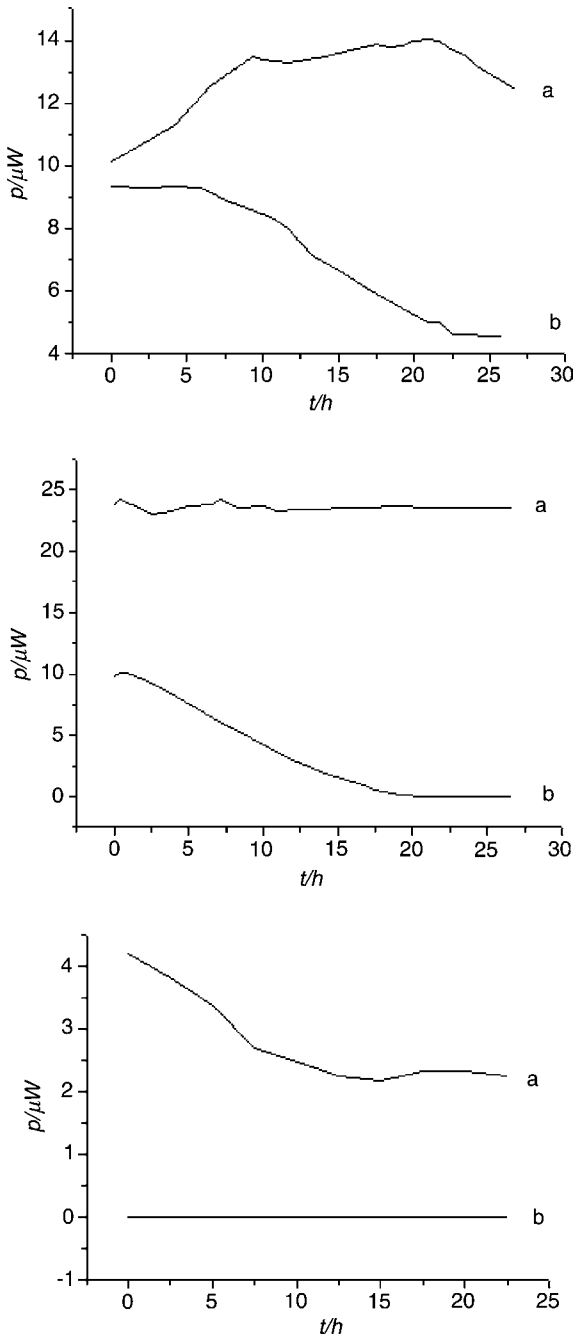

Fig. 3. The thermogenesis curves of cell line at different temperatures at (3.1) 37.0 °C; (3.2) 40.0 °C; (3.3) 43.0 °C; (a) control, (b) treated with 10 μg ml^{-1} $Cu^{II}(OP)_2$.

3.4. Flow cytometry assay

As shown in Fig. 6, DNA content frequency distribution histograms clearly illustrate that exposure of cells to hyperthermia and $Cu^{II}(OP)_2$ resulted in the appearance of cells with a fractional DNA content, typical of apoptosis. It formed a well-defined 'sub-G_1' peak which represents cells with lower DNA content. And the number of cells in "sub-G_1" can provide information on the number of apoptotic cells [14]. Fig. 6 shows the apoptosis percentage (AP) defined as the number of cells in sub-G_1 in total number of cells. AP increased from 1.01 in the control at 37.0 °C to 2.47 at 40.0 °C, and when the temperature reached 43.0 °C AP became 87.27. The results are consistent with the ones from microcalorimetry and fluorescence microscope. It indicated hyperthermia can lead to apoptosis in Bel-7402. When the cells treated with hyperthermia and $Cu^{II}(OP)_2$, AP significantly increased to 93.47, which was more than the one of the control at 43 °C. It was shown that hyperthermia and $Cu^{II}(OP)_2$ have the synergistic effects on Bel-7402.

4. Discussion

4.1. Relation between metabolic activity and apoptosis

Apoptosis is the prevalent form of programmed cell death that contributes to a number of human diseases [20]. The inhibition of apoptosis is implicated in diseases such as cancer, autoimmune disorders and viral infections. It is a type of cell death with distinct morphological and biochemical features when compared with necrosis. Only apoptotic cells display fragmentation of DNA, which would form the "sub-G_1" peak. And at the end of apoptosis, the cell is broken into multiple apoptotic bodies that are phagocytosed by neighboring cells. Because cellular contents are not released, this occurs with little inflammation. Therefore the importance of apoptosis in physiology and pathology is now widely recognized.

Apoptosis can be triggered by a great variety of chemical or physical agent. It is induced by such stimuli as growth factor withdrawal, oxidative stress, hypothermia, DNA damaging reagents, and antitumor drugs.

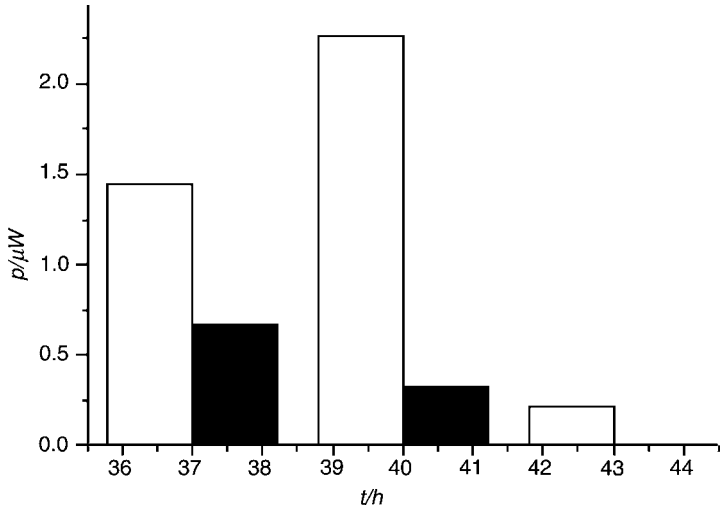

Fig. 4. The total metabolic heat released of cell line Bel-7402 at different temperature with (black column) or without (hollow column) $Cu^{II}(OP)_2$ during the experiment time.

The metabolic activity of apoptotic cells is lower than that of survival cells. Usually, treated cells were arrested in cell cycle and then apoptosis. The synthesis of protein and RNA were slowed or blocked. The total energy (Q) measured by microcalorimetry confirmed the difference between the metabolic energy of apoptotic cells and that of normal cells [21]. The results from the microcalorimetric study and cell researches also clarify the relation between metabolic activity and apoptosis.

4.2. ROS may play an important role in the mediation of apoptosis induced by hyperthermia and $Cu^{II}(OP)_2$

Reactive oxygen species (ROS) have been demonstrated to be mediators of cellular injury. A number of biochemical and physiological events associated with hyperthermia can potentially promote free radical formation [22]. Additional evidences indicate that heat increases the flux of cellular free radicals and support the hypothesis the increased generation of oxygen-centered free radicals and the resultant oxidative stress may mediate partially, heat-induced cellular damage [23].

In vitro studies on human monocytes/macrophages have shown that exposure of these cells to oxidants results in the production of the classical heat shock proteins and of oxidation-specific stress protein such as heme oxygenase or superoxide dismutase [24].

Furthermore, $Cu^{II}(OP)_2$ is known to promote hydroxyl radical formation from molecular oxygen. DNA fragmentation was detected in different cells treated with $Cu^{II}(OP)_2$, which was considered to result from direct attack upon DNA by the hydroxyl radical [2].

Based on the researches, ROS may play an important role in the mediation of apoptosis induced by hyperthermia and $Cu^{II}(OP)_2$.

4.3. The combination of hyperthermia and $Cu^{II}(OP)_2$ induced apoptosis in Bel-7402

Experimental studies have shown that hyperthermia artificial raising of temperature to 41–45.0 °C is an effective method of killing cells, especially for cells in low-pH and nutrient-deprived environments, conditions that are specifically found in malignant tumors [25].

Unlike the cell line K-562, of which the metabolic activity decreased when the temperature increased [26], the metabolic activity of cell line Bel-7402 has the maximal thermal power at 40.0 °C. From Fig. 4, it was found the metabolic activity of Bel-7402 cultured at 40.0 °C was even higher than that of

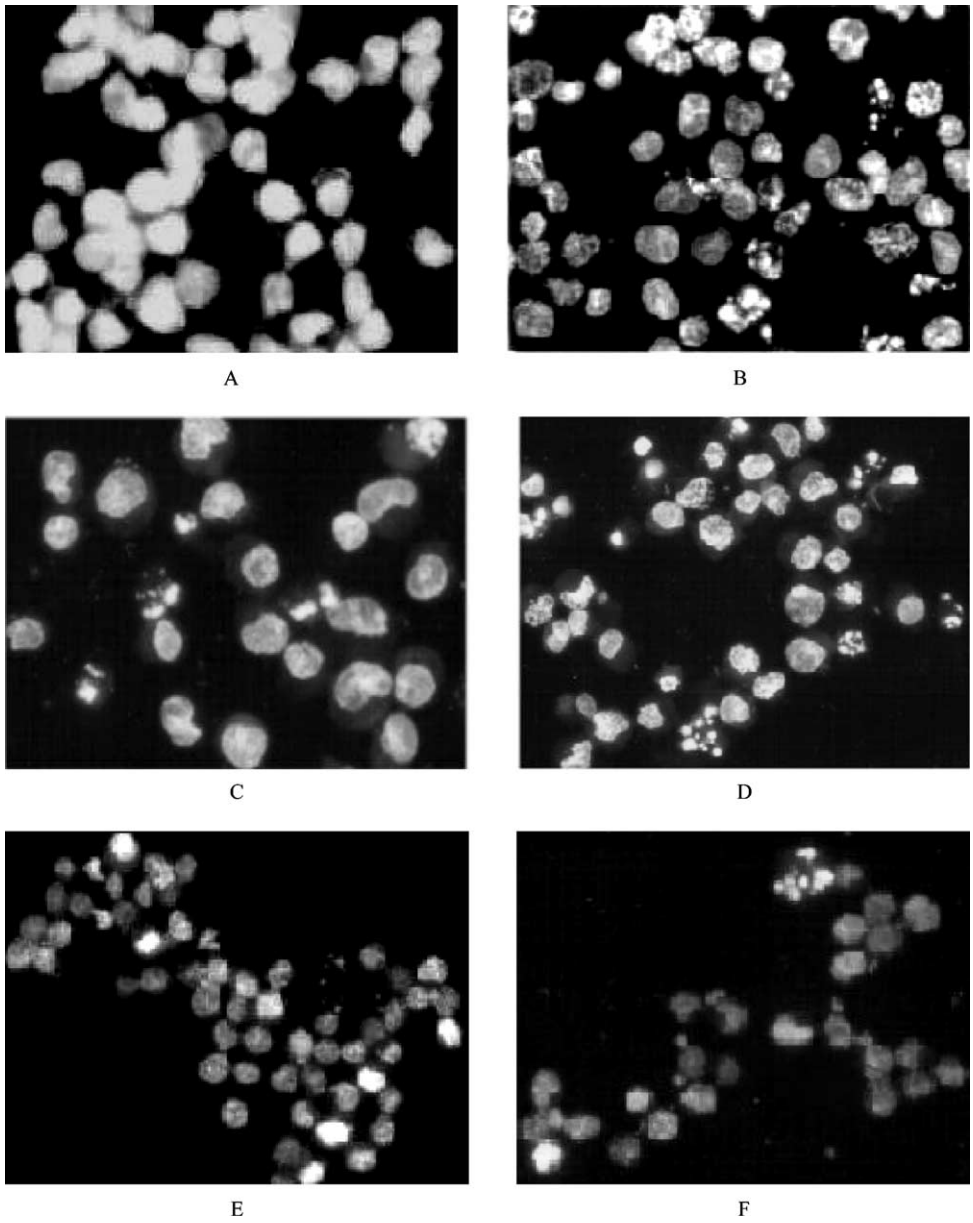

Fig. 5. The morphology of nucleus in Bel-7402 examined in a BH-2 fluorescence microscope using a standard fluorescein filter set (520 ± 20 nm). The magnification was set at 200× (A) control at 37.0 °C; (B) cells treated with $10\,\mu g\,ml^{-1}$ $Cu^{II}(OP)_2$ at 37.0 °C; (C) control at 40.0 °C; (D) cells treated with $10\,\mu g\,ml^{-1}$ $Cu^{II}(OP)_2$ at 40.0 °C; (E) control at 43.0 °C; (F) cells treated with $10\,\mu g\,ml^{-1}$ $Cu^{II}(OP)_2$ at 43.0 °C.

cells at 37.0 °C. While at 43.0 °C, however, the metabolic activity of cell line Bel-7402 was very low, suggesting that hyperthermia still has effect on Bel-7402.

When treated with hyperthermia and $Cu^{II}(OP)_2$, the metabolic activities of cells were inhibited. The inhibitory ratio of copper-1,10-phenanthroline combined with hyperthermia increased from 53.64 to

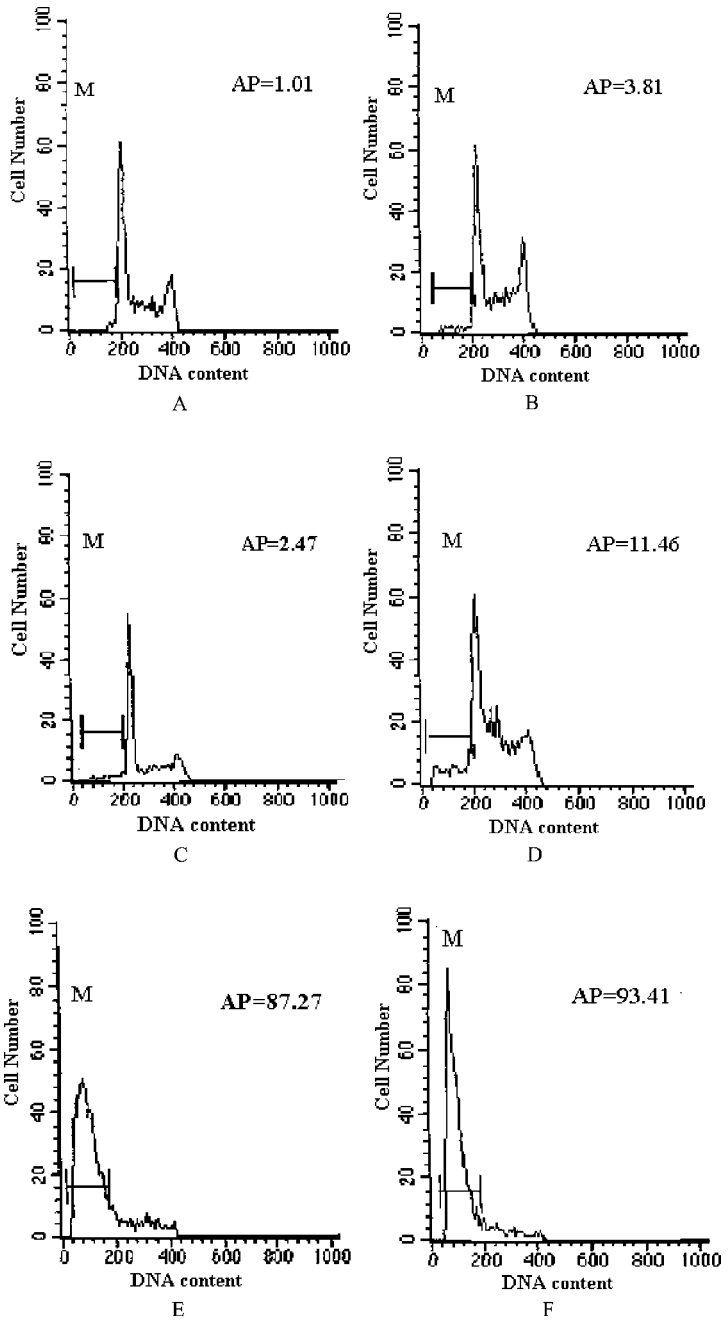

Fig. 6. DNA content analysis on FACSort when the cells cultured under different conditions, "M" is the marked region representing sub-G_1 peak: (A) control at 37.0 °C; (B) cells treated with 10 μg ml^{-1} $Cu^{II}(OP)_2$ at 37.0 °C; (C) control at 40.0 °C; (D) cells treated with 10 μg ml^{-1} $Cu^{II}(OP)_2$ at 40.0 °C; (E) control at 43.0 °C; (F) cells treated with 10 μg ml^{-1} $Cu^{II}(OP)_2$ at 43.0 °C.

100%. As a contrast, the inhibitory ratios are not such high when cells treated with hyperthermia and $Cu^{II}(OP)_2$ alone. The death-inducing effect was enhanced largely by the combination.

It was clear, from Figs. 5 and 6, that the cells treated with $Cu^{II}(OP)_2$ and hyperthermia died from apoptosis. Moreover, flow cytometry is the tool used in the quanlitification and quantification of apoptosis. When cells were treated with $Cu^{II}(OP)_2$ at 37.0 °C, AP was 3.81%. While treated with $Cu^{II}(OP)_2$ at 43.0 °C, 93.41% of the cells apoptosis.

From all of the results, we can conclude that the hyperthermia and $Cu^{II}(OP)_2$ induced apoptosis in Bel-7402 cell line and the combination would have synergistic effects.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant nos. 39770200 and 39730270).

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