Abstract

Background. Hyperthermia is one of the new and still poorly known methods used in cancer treatment. It consists of raising the patient’s body temperature for therapeutic purposes. The article presents the results of in vitro studies describing the effect of an elevated temperature of 39.5°C, the busulfan cytostatic and their combination on the level of apoptosis of human leukemia HL-60 cells.

Objectives. During the experiments, the influence of a 2.5°C temperature increase on the behavior of the population of 2 groups of HL-60 cells, with busulfan cytostatic and without the cytostatic, was investigated. The control group consisted of 2 groups of HL-60 cells incubated at 37.0°C with the cytostatic and without the cytostatic. Two questions were asked: 1. Is low-temperature hyperthermia likely to have an effect on the effectiveness of busulfan cytostatic? 2. Does the increase in temperature by 2.5°C have an effect on the level of apoptosis in the unsaturated HL-60 cell line?

Material and methods. Human promyelocytic leukemia cell line HL-60 was used in the experiments to examine the influence of temperature on apoptosis HL-60 in 2 separated incubators set to 37.0°C and 39.5°C for 3 h. Apoptosis was assessed with flow cytometry using Annexin V.

Results. An increase in mortality of HL-60 cells was found in the case of simultaneous exposure to elevated temperature and busulfan in comparison to the group of cells treated with the cytostatic alone. There was no observed effect of an elevated temperature of 39.5°C alone on the level of HL-60 cell apoptosis.

Conclusions. Analysis of the study results indicates that low-temperature hyperthermia may be used to increase the effectiveness of busulfan treatment. No effect of an elevated temperature of 39.5°C on the level of apoptosis in HL-60 cells that were not treated with busulfan was observed. There is a need to test the efficacy of other cytostatic agents at elevated temperatures.

Key words: apoptosis, in vitro, hyperthermia, busulfan, HL-60 cell line
Introduction

Due to the steady increase of cancer incidence, new ways of combating this group of diseases are being sought. The ways of fighting cancer can be divided into conventional and modern methods. Conventional methods of treatment include chemotherapy, radiotherapy and surgery. Modern methods of cancer treatment are the following: immunotherapy, gene therapy, hyperthermia, photodynamic therapy, and hormone therapy.\(^1\)

Hyperthermia is one of the new and still poorly known methods used in this field. It consists of raising the body temperature of a person for therapeutic purposes. Hyperthermia can be divided into low- and high-temperature hyperthermia, and due to its scope into regional tumor heating alone and global hyperthermia, which consists of raising the patient’s body temperature. Hyperthermia can be obtained using various methods, including radio waves, electromagnetic fields (the capacitance method), infrared radiation, microwaves, and thermal bath.

The electromagnetic wave can cause the body temperature to rise and at the same time can cause other physical processes, e.g., flow of the electrical charges in cancer tissue affected by this physical factor. Therefore, temperature rise is not the only factor influencing the processes taking place in the structures of neoplastic cells.

The question therefore is to what extent the temperature itself influences the apoptosis of cancer cells and what is the role of accompanying physical factors in this process.

To answer the first part of the question, we carried out experiments with a short term (3 h) temperature rise to 39.5°C (caused only by thermal conductivity based on the principle of heat exchange between bodies of different temperatures remaining in direct contact with each other, i.e., on the transfer of kinetic energy of random movement of particles as a result of their collisions, which leads to temperature equalization between bodies) on cultured HL-60 cells. In parallel, the influence of a 2.5°C temperature rise on apoptosis induced by busulfan treatment in relation to the effectiveness of this cytostatic activity at 37.0°C was also assessed.

Material and methods

Cell cultures

Human promyelocytic leukemia cell line HL-60 was used for the experiments. The cells were cultured in a fully humidified CO\(_2\) incubator (NuAire, Plymouth, USA) at 37°C, 5% CO\(_2\), in RPMI1640 cell medium (IITD PAN, Wroclaw, Poland) supplemented with 10% fetal calf serum (FCS; Invitrogen/Thermo Fisher Scientific, Warszawa, Poland) and 100 \(\mu\)g/mL of gentamicin (KRKA-Poland, Warszawa, Poland) culture medium. Twenty-four hours before starting the experiment, the cells were fed with an exchange of culture medium and the cell concentration was established at 3–4 × 10\(^5\)/mL.

To examine the influence of temperature on apoptosis, the HL-60 cells were suspended in fresh medium at a concentration of 2 × 10\(^5\)/mL with and without supplementation of 250 \(\mu\)g/mL of busulfan (Busilvex; Pierre Fabre Médicament, Paris, France), and incubated in 1 mL volume in 75 × 12 mm polystyrene culture tubes (Sarstedt, Warszawa, Poland) in 2 separated incubators set to 37.0°C (Hera Cell 150; Heraeus, Hanau, Germany) and 39.5°C (NuAire DH Autoflow CO\(_2\) Incubator) for 3 h.

Apoptosis measurement

Apoptosis was assessed with flow cytometry using an Annexin V binding test (Annexin V-FITC Apoptosis Detection Kit I; Becton Dickinson-Pharmingen, San Diego, USA), according to the protocol provided by the manufacturer. In brief, HL-60 cells after 3 h of incubation were washed once by centrifugation, suspended in Annexin V binding buffer and incubated for 15 min at room temperature with Annexin V-FITC and propidium iodide (PI) solutions. Apoptosis was measured in a PAS (Partec, Görlitz, Germany) flow cytometer. Fifteen thousand cells were assessed in each measurement. Apoptosis was the sum of cells stained with Annexin V-FITC only (Annexin V+) – early apoptosis – and cells stained both with Annexin V-FITC and PI (AnnexinV+/ Pl+) – advanced apoptosis.

Statistical analysis

The t-test and confidence intervals (CIs) were calculated using Microsoft Excel 10 (Microsoft Corp., Redmond, USA) to assess the statistical significance of the results obtained. The CIs were determined for the arithmetic mean of the test samples; they were calculated for a confidence level of 0.95. P-values less than 0.05 were considered statistically significant. The study assumes that the general population is normal. Normal decomposition testing was performed using the Shapiro-Wilk test.

Results

In the experiments carried out, the influence of a 2.5°C temperature increase on the behavior of the population of 2 groups of HL-60 cells, with busulfan cytostatic and without the cytostatic, was investigated. The control group consisted of 2 groups of HL-60 cells incubated at 37°C with the cytostatic and without the cytostatic. Two questions were asked:

1. Does the 2.5°C increase in temperature have an effect on the level of apoptosis of cultured HL60 cells?
2. Is low-temperature hyperthermia likely to have an effect on the effectiveness of busulfan cytostatic treatment?
The study was conducted in 2 incubators. In the 1st incubator, the temperature was 37.0°C and in the 2nd one, the cells were subjected to a temperature of 39.5°C. The test time for each of the populations studied was 3 h.

The experiment was repeated 9 times. As the number of repetitions (sample size) was less than 30, normality verification of distribution was performed with the non-parametric Shapiro-Wilk test, which verifies the null hypothesis that the sample originates from normal distribution. At the significance level of 0.05, the null hypothesis was not rejected because the calculated p-value = 0.6079 for the test statistics W = 0.9551 was higher than 0.05. Therefore, there were no grounds for rejecting the hypothesis of normal distribution.

Spontaneous apoptosis of HL-60 cells at 37°C and 39.5°C was less than 3% and did not differ considerably between these 2 groups. Early apoptotic cells (Annexin V+/PI−) consisted of 63% of both apoptotic populations. There was a statistically significant increase of apoptosis (p = 0.0198) in the busulfan-treated population at 39.5°C compared to the busulfan-treated population at 37.0°C. The test results are shown in Table 1 and Fig. 1. The majority of apoptotic cells were in the early phase of apoptosis: 82% at 37.0°C and 88% at 39.5°C, as could be expected from the relatively short exposure time to the drug (3 h).

In the experiments conducted, photographs of particular groups of cells examined were also taken, illustrating their state at the end of the experiment (Fig. 2). No morphological signs of apoptosis were seen in the control populations (Fig. 2A,B), whereas prominent apoptotic bodies were observed in both populations of HL-60 cells exposed to busulfan treatment (Fig. 2C,D).

**Discussion**

The potential of low-temperature hyperthermia in cancer chemotherapy is a new, promising area, bringing hope for new treatment options for this type of disease. The literature analysis suggests that the action of low-temperature hyperthermia may have an impact on the effectiveness of cytostatics.2 The most invasive form of hyperthermia is the local effect on the patient’s body with a very high temperature (>60°C), which can destroy (in a way – to “boil”) the cancer, which is called ablation.3 It has also been shown that the natural reaction of the human body manifested by the rise in temperature is a positive response of the patient’s body to the infection. It shortens the duration of the illness and increases the likelihood of survival.4 Some researchers point to the relationship between fever caused by infection and a simultaneous retreat of cancer.5

There are reports that hyperthermia induced by Whole Body Hyperthermia (WBH) fever affects the activation of T lymphocytes.6,7 It has also been shown that this type of hyperthermia affects the production and secretion of cytokines.8

Other researchers have shown that the combination of curcumin and hyperthermia has a beneficial anti-cancer effect and indicated that combination therapy significantly inhibited cell proliferation of MS-1 and LL/2 in vitro. It has also been shown that the combination therapy conducted in this way inhibited tumor growth and lengthened life expectancy.9 There are also reports from clinical studies that the rise of the temperature in the tumor tissue has positive therapeutic effects (e.g., recurrent breast and melanoma cancer) and increases the chances of survival in cases

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**Table 1.** Levels of apoptosis of HL-60 cell population as measured using flow cytometry. Values for control groups incubated at 37.0°C and 39.5°C, respectively, and the test groups (exposed to busulfan) at the same temperatures.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>37.0°C – control</th>
<th>39.5°C – control</th>
<th>37.0°C with busulfan</th>
<th>39.5°C with busulfan</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m [%]</td>
<td>1.94</td>
<td>1.89</td>
<td>9.20</td>
<td>20.49</td>
</tr>
<tr>
<td>SD [%]</td>
<td>1.14</td>
<td>0.98</td>
<td>4.57</td>
<td>8.04</td>
</tr>
<tr>
<td>CI [%]</td>
<td>0.62</td>
<td>0.51</td>
<td>2.49</td>
<td>4.38</td>
</tr>
</tbody>
</table>

n – number of experiments in the particular group (sample size); m – average value; SD – standard deviation; CI – confidence interval.
such as metastases to the lymph nodes and neck, glioma and cervical cancer.²

It has also been shown that raising the temperature to 39.5°C increases the expression of the Toll-like 4 receptor (TLR4) on human macrophages and causes increased cytokine production.¹⁰ Cancer cells exposed to elevated temperatures between 39.0°C and 45.0°C can be damaged or killed with minimal damage to normal tissues.¹¹

The examples presented above show that there is still a need to carry out a number of studies aimed at identifying the most efficient and optimal procedures of dealing with these issues using hyperthermia. This is due to the scarce knowledge of biological mechanisms that cause the potential anticancer effects of hyperthermia. For these reasons, we have carried out studies to observe the effect of 2.5°C elevated temperature on the apoptosis of HL-60 cells treated with busulfan.

Our results showed statistically significant differences in apoptosis level between the control group and test group only in the case of cells subjected to busulfan. HL-60 cells cultured at 39.5°C without the cytostatic showed even a slight decrease of apoptosis in comparison to the control group (but statistically not significant).

The increase in the mean apoptosis level of cells exposed to a temperature of 39.5°C and busulfan was more than 11 percentage points, which means an increase in cell mortality by 223% as compared to the cells incubated at 37.0°C. The results obtained and the analysis of publications in this field suggest that the use of low-temperature hyperthermia in the cytostatic area may increase its effectiveness. For this reason, there is a real need to continue such experiments and to test the effectiveness of other cytostatic agents at elevated temperatures as well as the effects of busulfan at higher temperatures. This research could, in the future, allow the introduction of new, more effective cancer treatment procedures utilizing chemotherapy. A particularly interesting option is the local heating of a tumor, aimed at increasing the local cytotoxicity of applied chemical compounds. This local action may increase the level of apoptosis of tumor cells caused by cytostatic treatment with a relatively minor negative effect on the rest of the human body.

Fig. 2. May-Grünwald-Giemsa-stained HL-60 cells after 3-hour culture without the cytostatic drug at 37.0°C (A) and 39.5°C (B) and cells cultured in the presence of busulfan at the same temperature values (C and D, respectively)
Continuation of research in this direction may show the efficacy of cytostatic drugs as a function of temperature rise, which may influence the determination of new, effective treatment procedures for cancer diseases with these chemical compounds, and the selection of cytostatic concentration applied depending on the current temperature of the patient’s body.

Conclusions

The use of low-temperature hyperthermia combined with chemotherapy in cancer treatment is a new area that requires further thorough research. The study confirmed that a 2.5°C temperature increase may be used to improve the effectiveness of busulfan treatment. The same hyperthermia does not influence the apoptosis of cells not exposed to the drug. There is a need to test the efficacy of other cytostatic agents at elevated temperatures, which may influence the determination of new, effective treatment procedures for cancer diseases with these chemical compounds, and the selection of cytostatic concentration applied depending on the current temperature of the patient’s body.

References
