INTRODUCTION

Cellular proliferation is arranged primarily by regulation of the cell cycle, which consists of four distinct sequential phases (G0/G1, S, G2 and M). Cell cycle coordination takes place mainly at G1/S and G2/M transitions at the various checkpoints. Control of cell division is a tightly regulated process involving a family of proteins that have variable expression according to the stage of the cell cycle\textsuperscript{1-3}. At first presentation of childhood acute lymphoblastic leukemia (ALL), the prognostic value of the proliferative capability of lymphoblasts remains controversial, and there is no clear evidence of an association with prognosis. However, lymphoblasts with high proliferative potential are sensitive to chemotherapeutic drugs targeting the cell cycle\textsuperscript{4,5}.

Heparin has shown anticoagulant, antihypertensive, anti-inflammatory and antiproliferative activities\textsuperscript{6,8}. These activities depend on its complicated structure. Heparin has been shown to be a potent inhibitor of the proliferation of several cell types, including vascular smooth muscle cells (VSMC), renal mesangial cells, certain types of fibroblasts, hepatoma cells and cervical epithelial cells\textsuperscript{9,12,13}. Heparin binds tightly to specific SMC surface receptors, accumulates within the cell, and blocks a critical point in the G0/G1 to S transition of the cells\textsuperscript{10,11}. Despite the well-documented antiproliferative effect of heparin on VSMC, the molecular mechanisms that are involved are not yet fully understood (12). Heparin causes apoptosis of human peripheral blood neutrophils, lymphoblasts and mononuclear cells, and indicates its apoptotic effect on lymphoblasts via an extrinsic pathway of apoptosis\textsuperscript{14-17}.

In this \textit{in vitro} study, we investigated the effect of heparin on the cell cycle of lymphoblasts in newly diagnosed ALL patients by flow cytometry (FCM) and tried to establish which phase in the cell cycle of the lymphoblasts can be considered to be heparin-sensitive.
RESULTS

The cell cycle analyses were performed in different heparin concentrations (0, 10 and 20 U/mL) at 0, 1, and 2h after adding heparin on the lymphoblasts.

The mean percentage of blast cells in the G0/G1 phase in different heparin concentrations at 0, 1, and 2h are shown in Table 1. In 0 U/mL heparin concentration, the mean percentage of blast cells in the G0/G1 phase at 2h was significantly lower than those at 0h and 1h (P<0.000). The highest percentage of blast cells in the G0/G1 phase was established in 0 U/mL heparin concentrations at 0h. The lowest percentage of blast cells in the G0/G1 phase was determined in 20 U/mL heparin level at the first hour. The mean percentage of blast cells in the G0/G1 phase in 20 U/mL heparin concentration at 1h was significantly lower than in 0 U/mL and 10 U/mL heparin concentrations at 1h (P<0.000), and in 20 U/mL heparin concentration at 0h (P<0.000). There were significant differences between the percentage of the lymphoblasts in 20 U/mL, 10 U/mL, and 0 U/mL heparin concentrations at 2h (P<0.000).

The mean percentage of blast cells in the G2/M phase in the different heparin concentrations at 0, 1, and 2h are shown in Table 2. There were significant differences in the mean percentage of blast cells in the G2/M phase in 0 U/mL heparin concentration at 0, 1, and 2h (P<0.001). The lowest percentage of blast cells in the G2/M phase was determined in 0 U/mL heparin concentration at the second hour. The highest percentage of blast cells in the G2/M phase was determined in 20 U/mL heparin level at the first hour. The mean percentage of blast cells in the G2/M phase in 20 U/mL heparin concentration at 1h was significantly higher than those in 0 U/mL and 10 U/mL heparin concentrations at 1h (P<0.000), and in 20 U/mL heparin concentration at 0h (P<0.003). The mean percentage of blast cells in 20 U/mL heparin levels at 1h was significantly higher than those in 0 U/mL and 10 U/mL heparin levels (P<0.000).
The mean percentage of blast cells in the S phase in different heparin concentrations at 0, 1, and 2h are shown in Table 3. The mean percentage of blast cells in the S phase in 0 U/mL heparin concentration was the same at 0, 1, and 2h. The lowest percentage of blast cells in the S phase was determined in 0 U/mL heparin concentration. The highest percentage of blast cells in the S phase was determined in 20 U/mL heparin concentration at the first hour. The mean percentage of the blast cells in the S phase in 20 U/mL heparin concentration at 1h was significantly higher than in 0 U/mL and 10 U/mL heparin concentrations (P<0.000, P<0.006, respectively) and in 20 U/mL heparin concentration at 0h (P<0.000). There were significant differences between the percentages of the blast cells in 20 U/mL and 10 U/mL, and 20 U/mL and 0 U/mL heparin levels in S phase at 1h (P<0.000), and the highest percentage of the lymphoblasts in S phase at 2h was detected in 20 U/mL heparin levels.

Prominent alterations in the cell cycle distribution were observed in 20 U/mL heparin concentration at 1h. There was a movement of cells from G0/G1 to G2/M and S phases. The percentage of the lymphoblasts in the G0/G1 phase was decreased while the percentage of the lymphoblasts in the G2/M and S phases was arrested in 20 U/mL heparin levels at 1h.

**DISCUSSION**

Cell division is under the control of a family of proteins, termed cyclin and cyclin-dependent kinases (CDKs) involved in signal transduction of the cells. The balance between CDK activation and inactivation determines that the cells proceed through G1 into S phase and from G1 to M. It is widely accepted that the activation of checkpoints in response to DNA damage leads to the cell cycle; however, in the case of severe damage, the cell cycle arrest leads to apoptotic cell death. In tumor cells, DNA damage principally leads to the cell cycle arrest in the G2 phase.

Cell death occurs via two different mechanisms: necrosis and apoptosis. Apoptosis is a form of cell death widely observed in nature that plays an important role in embryogenesis,
normal tissue turnover, tumorigenesis and elimination of damaged cells. Intrinsic factors related to chemical or physical cell damage can initiate apoptosis in a specific cell cycle phase. It is generally believed that apoptosis follows two major distinct pathways, the death receptor-mediated pathway, associated with caspase-8 activation (extrinsic apoptosis), and the stress-induced mitochondrial-dependent pathway, associated with caspase-9 activation (intrinsic apoptosis)\textsuperscript{15,20-22}.

Leukemia cells were found sensitive to chemotherapeutic agents that either interfere with the cell cycle or cause apoptosis. Some chemotherapeutic agents cause cell death through interfering with the processes of the cell cycle, while others cause cell death by apoptosis, which plays an important role in the balance between cell replication and cell death\textsuperscript{5,23}. Many chemotherapeutic agents are effective on the cells that are in the S phase of the cell cycle. For example, artemisinin mainly arrests the cell cycle in the G1 phase, while paclitaxel and lycorine were shown to arrest the cell cycle in the G2/M phase and induce apoptosis by increasing the activation of caspase-8, caspase-9, or caspase-3\textsuperscript{24}. Similarly, an increase in the percentage of cells in the G2/M phase upon treatment with sulforaphane was observed in HT29 human colon cancer cells as well as in Jurkat T-leukemia\textsuperscript{25}.

Heparin has shown to have various effects on peripheral blood cells, lymphoblasts, fibroblasts, hepatoma cells, VSMC, renal mesangial cells and cervical epithelial cells\textsuperscript{9-16}. Heparin blocks through the G0/G1 phase to the S phase transition of VSMC\textsuperscript{11}. In human peripheral blood neutrophils, mononuclear cells and lymphoblasts, heparin induces apoptosis\textsuperscript{14-17}.

Manaster et al.\textsuperscript{14} demonstrated in an \textit{in vitro} study that heparin in concentrations of 50-200 U/mL is able to induce apoptosis in human peripheral neutrophils. Erduran et al.\textsuperscript{15} first determined the apoptotic effect of heparin in concentrations of 5, 10 and 20 U/mL on lymphoblasts in an \textit{in vitro} study, while it apparently did not affect neutrophils and
mononuclear cells. The highest apoptosis on the lymphoblasts was determined in 20 U/mL heparin level at 1h. In another *in vitro* study, the apoptotic effect of heparin in concentrations of 10 and 20 U/mL on the lymphoblasts and on Bcl-2 and Fas protein expression of the blasts were investigated by FCM. The highest Fas protein expression and the lowest Bcl protein-2 expression on the lymphoblasts and the highest apoptosis were found in 20 U/mL heparin concentration at 1h. These results were correlated with each other\textsuperscript{16}.

Recently Erduran et al.\textsuperscript{17} reported caspase-3 and caspase-8 activations of the lymphoblasts after incubation with heparin *in vitro*. They suggested that heparin induce apoptosis of the lymphoblasts and activation of caspase-3 and -8. In that study, the greatest apoptotic effect of heparin on the lymphoblasts was detected in 20 U/mL concentration at the first hour. These researches indicated that low-dose heparin caused significant levels of apoptosis of lymphoblasts, and apoptosis was found to increase with increased heparin levels\textsuperscript{15-17}.

In the present study, the percentages of blasts in G0/G1 phase decreased, whereas the percentages of blasts in the S and G2/M phases substantially increased in 20 U/mL with the heparin concentration at 1h. The greatest effect of heparin was determined in 20 U/mL concentration at the first hour in G0/G1, G2/M and S phases. We have demonstrated that heparin affects the cell cycle of lymphoblasts in a dose- and time–dependent manner. We investigated whether the apoptotic effect of heparin was able to affect the cell cycle of lymphoblasts. The antiproliferative effect of heparin on the lymphoblasts was mediated by cell cycle arrest, and the prolonged G2/M and S phases could play a key role favoring cell cycle progression and proliferation. These findings provide new insights into the molecular mechanism of the apoptotic effect of heparin.

It was suggested that the lymphoblasts percentage in the S phase is linked to outcome in childhood ALL. Lymphoblast count higher than 6% in the S phase was associated with a
significantly short remission period. In our study, the percentage of the cells in G0/G1 phase decreased, while the percentage of cells in the G2/M and S phases increased after the lymphoblasts were incubated with heparin in 10 and 20 U/mL concentrations at 1 and 2h. These findings indicate an arrest of the lymphoblast cell cycle at the G2/M and S phases by heparin.

In conclusion, heparin induces apoptosis of lymphoblasts to arrest the cell cycle in the G2/M and S phases. With regard to the effect of heparin, we did not investigate which molecular pathway is involved in the cell cycle of lymphoblasts. We believe that the relationship between heparin cell cycle phases and apoptosis needs further studies at the molecular level. The lymphoblasts arrested in S phase by low-dose heparin become more sensitive to apoptosis.

METHODS

Twelve children (8 girls, 4 boys; aged between 2 and 15 years) with newly diagnosed ALL (all had B-cell leukemia) were included in the study with written consent of their parents. Diagnosis of the patients was done according to the finding of completed blood counts, peripheral and bone marrow aspiration (BMA) smears, histochemical staining of BMA smears, and FCM analysis (Goulter Epics Elite ESP Flow Cytometry) of BMA materials.

Cell typing

CD3 PE (Coulter PN IM1282), CD7 FITC (Coulter PN IM0585), CD10 FITC (Coulter PN IM0471), CD13 FITC (Coulter PN IM0778), CD14 FITC (Coulter PN IM0650), CD19 FITC (Coulter PN IM1284), CD20 FITC (Coulter PN IM1455), CD33 FITC (Coulter PN IM1179), CD45 FITC (Coulter PN IM0782) and MPO FITC (Coulter PN IM1874)
monoclonal antibodies were used for the diagnosis of ALL. All patients had monoclonal antibodies positivity for B-cell leukemia.

**Separation of blast cells**

Bone marrow aspiration materials were drawn into a tube with ethylene diamine tetraacetate (EDTA). The same quantities of phosphate-buffered saline (PBS) and the BMA sample were added into the tube. The sample was stirred, and waited for 30 min at room temperature. Buffy coat obtained from the upper surface of the specimen was added onto Ficoll-Hypaque 1077 (Lymphocyte separation medium Gibco BRL 13010-12, Grand Island, NY) and centrifuged at 700g for 30 min. Mononuclear cells containing ≥90 lymphoblasts were obtained from the upper surface of the specimen and washed twice with PBS to exclude debris. The blast cells were suspended at a concentration of 3-5x10⁵ cells/mL in RPMI with L-glutamine without sodium bicarbonate medium (Sigma R-6504, Miami, FL). 100 mL of each material was studied for immunotyping by FCM. The remaining amount of each sample was kept at -80°C until the study was performed.

Pure heparin was used in the study (Sigma Biochemicals and Reagents-2001 Catalog, Sigma H 3149). The purity and activity of heparin were Grade I, ≥140 USP unit/mg. The heparin did not include the additional stabilizing agents. The blast cell suspensions were thawed at room temperature. Different heparin concentrations (0, 10, 20 U/mL) were added on the blast cells (1 mL). Each tube included different heparin concentrations and was divided into 3 different tubes. All the samples were processed with a Coulter DNA-prep reagent kit (CN: 640445) at 0, 1, and 2 hours (h). The DNA-prep reagent kit contained DNA prep-LPR solution (<0.1% potassium cyanide, <0.1 sodium nitride, nonionic detergents, saline, and stabilizers) and the DNA-prep stain (50 μg/mL propidium iodide [PI], <0.5% NaN₃, saline, and stabilizers). The blast membranes were pored by the DNA-prep LPR solution, and RNAs and DNAs of the blasts were stained with PI. Stained RNAs were removed from the medium
by RNase; therefore, DNA content was marked by PI. The aliquots were taken following 0, 1, and 2h, and FCM analyses were carried out for cell cycle of the blast cells in a Coulter Epics Elite Flow Cytometer (Multicycle DNA, Phoenix Flow Systems, San Diego, CA 92121). The analysis at 0h was done immediately after (within 3-5 seconds) the addition of heparin on the blasts.

Flow cytometric analyses could not be performed in 10 and 20 U/mL heparin concentrations at 3h because the samples were seen to have transformed into a gelatinous substance. A gelatinous substance similarly developed following the addition of higher heparin concentrations (30 and 50 U/mL) into the lymphoblasts samples.

The percentages of the cells in the G0/G1, G2/M, and S phases were determined from an analysis by a computer program called PEAK, generously provided by Dr. Phillip Dean\textsuperscript{27}.

**Statistical analysis**

Data obtained from FCM and fluorometric analyses were analyzed by the SPSS version 10.0 package statistical programs. The appropriateness of the data to normal distribution was determined by Kolmogorov-Smirnov test. Paired \( t \)-test (post \textit{hoc}) was used to determine statistically the significant differences between the measurements using different heparin concentrations. Results were calculated as arithmetic mean ± standard deviation (X±SD).
REFERENCES


Table 1: G0/G1 PHASE: PERCENTAGES OF LYMPHOBLASTS DETECTED BY FLOW CYTOMETRY IN 0, 10, AND 20 U/ML HEPARIN CONCENTRATIONS AT 0, 1, AND 2 HOURS

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Time (hour)</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 U/ml</td>
<td>10 U/ml</td>
<td>20 U/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>X±SD</td>
<td>X±SD</td>
<td>X±SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>min-max</td>
<td>min-max</td>
<td>min-max</td>
</tr>
<tr>
<td>0 U/ml</td>
<td></td>
<td>97.80 ± 0.51a</td>
<td>97.72 ± 0.47d</td>
<td>97.62 ± 0.48g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97-98.5</td>
<td>97-98.3</td>
<td>96.9-98.2</td>
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<tr>
<td>10 U/ml</td>
<td></td>
<td>85.38 ± 4.3b</td>
<td>85.54 ± 2.06c</td>
<td>90.77 ± 0.81h</td>
</tr>
<tr>
<td></td>
<td></td>
<td>78.9 - 92</td>
<td>82.4 - 89</td>
<td>90 – 92</td>
</tr>
<tr>
<td>20 U/ml</td>
<td></td>
<td>88.38 ± 3.12c</td>
<td>76.91 ± 4.58f</td>
<td>78.34 ± 2.12i</td>
</tr>
<tr>
<td></td>
<td></td>
<td>83.3 ± 92</td>
<td>69.6 - 84.5</td>
<td>74.9 - 81</td>
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</tbody>
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a-b, a-c, a-g, c-f, c-i, d-g, d-e, d-f, e-h, e-f, g-h, g-i, h-i: p<0.000, a-d: p<0.002, b-h: p<0.001
Table 2: G2/M PHASE: PERCENTAGES OF LYMPHOBLASTS DETECTED BY FLOW CYTOMETRY IN 0, 10, AND 20 U/ML HEPARIN CONCENTRATIONS AT 0, 1, AND 2 HOURS

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Time (hour)</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 U/ml</td>
<td>X±SD</td>
<td>1.16</td>
<td>1.18</td>
<td>1.08</td>
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<tr>
<td></td>
<td>min-max</td>
<td>0.5-1.9</td>
<td>0.5-1.9</td>
<td>0.4-1.8</td>
</tr>
<tr>
<td>10 U/ml</td>
<td>X±SD</td>
<td>7.24</td>
<td>4.50</td>
<td>3.00</td>
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<tr>
<td></td>
<td>min-max</td>
<td>4.1-10.1</td>
<td>1.8-7.4</td>
<td>1.3-4.0</td>
</tr>
<tr>
<td>20 U/ml</td>
<td>X±SD</td>
<td>6.00</td>
<td>10.21</td>
<td>9.37</td>
</tr>
<tr>
<td></td>
<td>min-max</td>
<td>3.0-9.0</td>
<td>5.4-18.4</td>
<td>4.9-13.0</td>
</tr>
</tbody>
</table>

a-b, a-c, a-g, b-c, b-h, d-f, e-f, g-i, h-i: p< 0.000, c-f: p<0.003, d-g: p<0.001, d-e: p<0.003, c-i, e-h: p<0.006, g-h: p<0.019
Table 3: S PHASE: PERCENTAGES OF LYMPHOBLASTS DETECTED BY FLOW CYTOMETRY IN 0, 10, AND 20 U/ML HEPARIN CONCENTRATIONS AT 0, 1, AND 2 HOURS

<table>
<thead>
<tr>
<th>Heparin</th>
<th>X±SD</th>
<th>min-max</th>
<th>X±SD</th>
<th>min-max</th>
<th>X±SD</th>
<th>min-max</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 U/ml</td>
<td>1.03 ± 0.60a</td>
<td>0.2-2.0</td>
<td>1.03 ± 0.60d</td>
<td>0.2-2.0</td>
<td>1.03 ± 0.60g</td>
<td>0.2-2.0</td>
</tr>
<tr>
<td>10 U/ml</td>
<td>7.43 ± 3.61b</td>
<td>3.0 - 14.4</td>
<td>10.12 ± 2.71c</td>
<td>7.0 - 15.3</td>
<td>6.22 ± 0.95h</td>
<td>5.0 - 7.9</td>
</tr>
<tr>
<td>20 U/ml</td>
<td>5.45 ± 2.31c</td>
<td>2.2 - 7.5</td>
<td>13.63 ± 3.47f</td>
<td>10.0 - 21.4</td>
<td>12.11 ± 2.73i</td>
<td>9.9 - 17.4</td>
</tr>
</tbody>
</table>

a-b, a-c, c-f, c-i, d-e, e-h, d-f, g-h, g-i, h-i: p<0.000; e-f: p<0.006