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RESEARCH

COMPARISON OF PERCENTAGE PERIPHERAL BLOOD LYMPHOBLAST PROLIFERATION AND APOPTOSIS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA BEFORE AND AFTER CHEMOTHERAPY INDUCTION PHASE

(Perbandingan Persentase Proliferasi dan Apoptosis Limfoblas di Darah Tepi di Pasien Leukemia Limfoblastik Akut Anak Sebelum dan Sesudah Kemoterapi Tahap Induksi)

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ABSTRAK

Leukemia Limfoblastik Akut (LLA) adalah penyakit neoplasma yang dihasilkan dari perpindahan somatik multistep progenitor limfoid di sumsum tulang, ditandai maturation arrest, proliferasi tidak terkendali seri limfoid serta penumpukan limfoblas di sumsum tulang dan darah tepi. Kelainan terkait aktivitas proliferasi sel berkaitan dengan kendali apoptosis. Penelitian ini bertujuan mengetahui perbandingan persentase proliferasi dan apoptosis limfoblas di darah tepi pasien LLA anak sebelum dan sesudah kemoterapi tahap induksi. Subjek penelitian sebesar 12 pasien LLA anak kasus baru yang diperiksa sebelum dan sesudah kemoterapi tahap induksi. Jenis penelitian ini cohort prospektif tanpa pembanding. Pemeriksaan proliferasi limfoblas dilakukan menggunakan spesimen darah tepi sedangkan pengecatannya menggunakan reagen PI/RNase. Pemeriksaan apoptosis limfoblas dilakukan menggunakan spesimen darah tepi sedangkan pengecatannya menggunakan reagen FITC Annexin V. Pembacaan proliferasi dan apoptosis limfoblas menggunakan alat BD FACSCallibur dengan metode flow cytometry. Rerata persentase proliferasi dan apoptosis limfoblas sebelum kemoterapi tahap induksi 7,84%±7,50 dan 11,50%±8,60 sesudah kemoterapi tahap induksi 3,2%±1,89 dan 13,42%±8,10. Persentase proliferasi limfoblas di darah tepi sesudah pemberian kemoterapi tahap induksi terdapat penurunan bermakna, sedangkan pemeriksaan apoptosis limfoblas didapatkan peningkatan yang tidak bermakna. Persentase proliferasi limfoblas di darah tepi sesudah kemoterapi tahap induksi terdapat penurunan bermakna, sehingga dapat dipergunakan sebagai peramal keberhasilan pengobatan pasien LLA anak. Pemeriksaan apoptosis limfoblas tidak terdapat perbedaan bermakna sebelum dan sesudah kemoterapi tahap induksi. Perlu penelitian lebih lanjut untuk menganalisis hasil yang didapat.

Kata kunci: *Proliferasi, apoptosis, limfoblas, leukemia limfoblastik akut anak*

ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is a neoplastic disease in the multistep lymphoid progenitor of the bone marrow, characterized by maturation arrest, uncontrolled proliferation of lymphocytes, lymphoblast accumulation in bone marrow and peripheral blood. Disorders related to the activity of cell proliferation are closely related to the control of apoptosis. The aim of this study was to compare the percentage of lymphoblast proliferation and apoptosis in peripheral blood of pediatric ALL before and after chemotherapy induction phase. Subjects were 12 new cases of pediatric ALL, examined before and after chemotherapy induction phase. This research was a prospective cohort without comparison. Lymphoblast proliferation examination was performed using peripheral blood specimens and staining by PI/RNase reagent, while lymphoblast apoptosis examination used staining by Annexin V FITC reagent. Examination of lymphoblast proliferation and apoptosis was done by BD FACSCalibur using flow cytometry. Mean percentage of lymphoblast proliferation and apoptosis before chemotherapy induction phase was 7.84%±7.50 and 11.50%±8.60, after

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chemotherapy induction phase 3.2%±1.89 and 13.42%±8.10. In the peripheral blood lymphoblast proliferation percentage decreased after chemotherapy induction phase compared to before in pediatric ALL. Examination of lymphoblast apoptosis showed no significant improvement. Percentage of lymphoblast proliferation in peripheral blood after chemotherapy induction phase decreases, so it could be used to predict therapeutic efficacy in pediatric ALL. Lymphoblast apoptosis examination showed no significant difference. Further research is needed to analyze these results.

Key words: Proliferation, apoptosis, lymphoblast, pediatric acute lymphoblastic leukemia

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is the most common malignancy in children, approximately 25% of all cases of malignancy in children. The incidence of leukemia in Indonesia 2.5–4.0 per 100,000 children is estimated to be 2000–2300 new cases every year.[1] The disease is characterized by maturation arrest, uncontrolled proliferation of bone marrow and lymphoid progenitor lymphoblast accumulation in the bone marrow and peripheral blood.[2]

The molecular process that regulates the cell cycle and mechanisms of malignant cells escaping the control of the cell cycle, as well as the cessation of proliferation associated lifetime of the cells, is the key to understanding normal and malignant cells. The cell cycle is a process that is organized regularly to copy and pass on genetic information from one generation to the next. Cell cycle or normal cell proliferation takes place through a cycle of four sequential phases, each referred to as phase G1 (first gap), S phase (DNA synthesis), G2 phase (gap 2) and M-phase (mitosis).[3]

Disorders related to the activity of cell proliferation are also closely related to the control of apoptosis. Failure to implement the mechanism of cancer cell apoptosis is one of the factors underlying the increasing growth of cancer, genetic instability of the cells in question and resistance to chemotherapy. The purpose of cancer therapy is to kill cancer cells and restore the impaired gene function, among others restore the apoptotic function. One indicator of the success of cancer therapy is the decreased proliferation and increased apoptosis of cancer cells. Research on cell proliferation has been performed by Ffrench and colleagues[4] in pediatric Acute Lymphoblastic Leukemia (ALL) showed a longer remission time when the level of G2+M phase is higher.[4] Research on apoptosis by Kaparou and colleagues[5] in pediatric ALL found increased levels of apoptosis in remission when compared to the current diagnosis by the expression of FAS (24.0±6.1% compared to 8.0±1.9%, p=0.035).[5] Until now, a study comparing the percentage of lymphoblast proliferation and apoptosis in pediatric ALL before and after induction phase of chemotherapy has not been studied in the Dr. Soetomo Hospital Surabaya.

The aim of this study was to compare and analyze the differences in the percentage of lymphoblast proliferation and apoptosis in peripheral blood before and after the induction phase of chemotherapy in pediatric ALL. This study is expected to be one of the indicators of the success of chemotherapy in pediatric ALL.

METHODS

The study was observational analytical with a prospective cohort design without comparison. This research was conducted in the Hemato-Oncology patient ward, Department of Pediatrics, Dr. Soetomo Hospital-Faculty of Medicine, University of Airlangga, as a determination of diagnosis and sampling. Sample processing (examination of lymphoblast proliferation and apoptosis) was conducted at the Laboratory of Clinical Pathology, Dr. Soetomo Hospital Surabaya. This study was conducted from February 2016 to April 2016. The subjects were patients in Hemato-Oncology Ward, Department of Pediatrics, Dr. Soetomo Hospital-Faculty of Medicine, University of Airlangga, newly diagnosed with Acute Lymphoblastic Leukemia and who met the inclusion criteria and no one in accordance with the exclusion criteria.

Inclusion criteria were patients with ALL aged 1 month to 18 years (newly diagnosed) and who underwent regular chemotherapy induction phase. The protocol of chemotherapy induction phase was based on ALL Protocol Indonesia 2013.[6]

Exclusion criteria were acute lymphoblastic leukemia patients who had received steroid therapy or cytostatic previously, as well as patients who experienced an infection (toxic granules found in blood smears and or shift to the left in a complete blood count).

Calculation of sample size was obtained by using the Lemeshow‘s formula so that the minimum sample required was 12 samples for each inspection of lymphoblast proliferation and apoptosis in pediatric ALL before and after induction phase of chemotherapy.

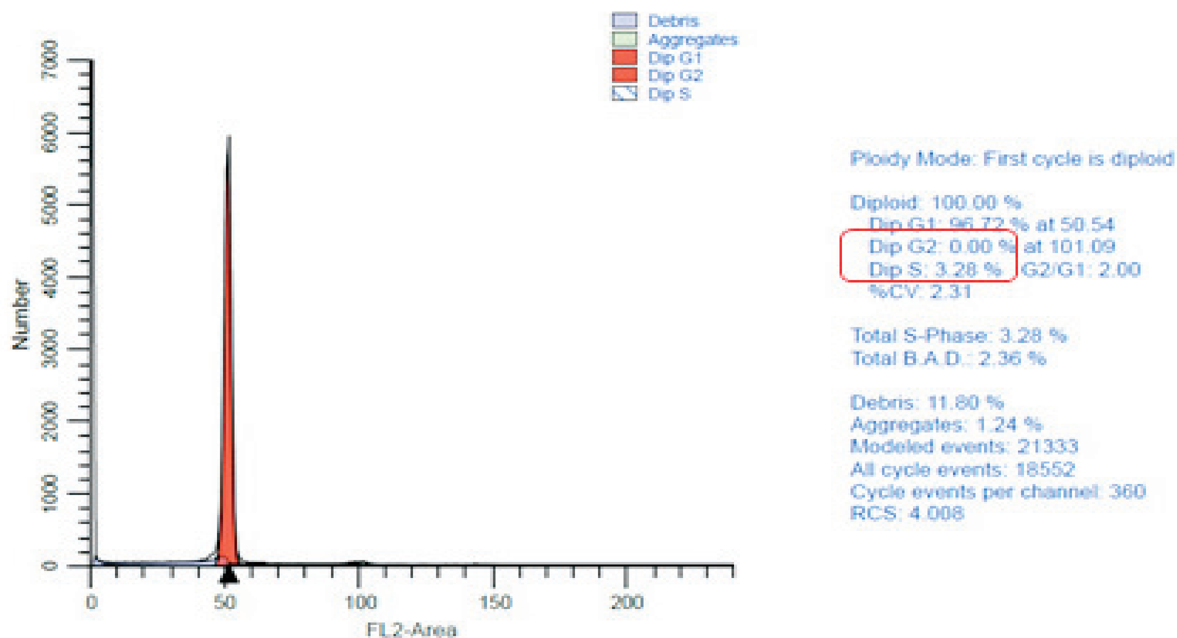

Figure 1. Proliferation pattern in scattergram.
The results were shown as the lymphoblast Proliferation percentage value G2 and S

Specimens used for the inspection lymphoblast proliferation and apoptosis was 3 mL venous blood with EDTA anticoagulant. Materials for examination of lymphoblast proliferation were PBS, Ficoll-Hypaque, cold 70% Ethanol, PI/RNase reagent. Materials for examination lymphoblast apoptosis were PBS, Ficoll-Hypaque, FITC Annexin V reagent and binding buffer. Tools used for specimen collection were paper alcohol swab, gloves and sterile syringes. Tools used to store the specimen were EDTA tube for storing specimen examination of lymphoblast apoptosis and proliferation, a refrigerator -20°C was also needed to store specimens of lymphoblast proliferation. Tools for examination of the proliferation and apoptosis were Valcon tubes, centrifuge tubes, micropipettes, swing centrifuge, Pasteur pipettes, FACS Calibur by flow cytometry method.

Examination of lymphoblast proliferation before chemotherapy induction phase used cells generated from PBMC of ALL patients who had never undergone chemotherapy induction phase and examined using PI staining by PI/RNase reagents and read using a flow cytometry method by BD FACS Calibur. Re-examination was done after patients underwent chemotherapy induction phase. Lymphoblast proliferation inspection unit was percentage of G2/M and S in the scattergram as seen in Figure 1.[7]

Lymphoblast apoptosis examination prior to chemotherapy induction phase was cells produced from PBMC ALL patients who had never undergone chemotherapy induction phase and was examined using Annexin V FITC reagent and performed by BD FACSCalibur using flow cytometry. Re-examination was done after patients underwent chemotherapy induction phase. Examination of lymphoblast apoptosis was expressed by percentage on the upper and lower right quadrant (upper right/UR) and lower right/LR) scattergram as seen in Figure II.2 and II.3.[8]

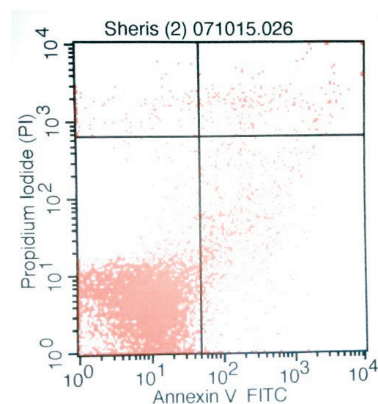

Figure 2. Apoptosis Pattern in scattergram.
The result at the upper and lower right quadrant showed the area of apoptosis

Tube: tube #1
Acquisition Date: 10-Sep-15
Gated Events: 6684
X Parameter: Annexin V FITC (Log)
Quad Location: 49, 644

Panel: Apoptosis
Gate: G1
Total Events: 10000
Y Parameter: Propidium Iodide (PI) (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	2	0.03	0.02	25.36	9.91	1680.76	1596.34
UR	70	1.05	0.70	1976.92	1164.92	1996.91	1731.83
LL	5624	84.14	56.24	10.31	6.85	5.15	3.67
LR	988	14.78	9.88	552.27	178.64	31.49	8.58

Figure 3. Apoptosis result.
Results circled in red were the percentages of apoptosis

RESULTS AND DISCUSSION

In the first examination, 37 new ALL patients who met the inclusion criteria were found, but during the time 10 patients died, 7 patients did not return so both were categorized as drop out criteria and 8 patients had a low sample volume or the process was less precise, so the results could not be analyzed. The final results were obtained in 12 patients who could be followed for the examination of both proliferation and apoptosis lymphoblasts after undergoing chemotherapy patients completed the induction phase.

Characteristics of the samples consisted of gender, age, hemoglobin, leukocyte count and platelet count as seen in Table 1.

Patients with pediatric ALL studied were mostly boys as many as 9 (75%), while 3 (25%) were girls. This was consistent with the literature which mentioned that ALL types of T-cell and B-cell were male dominated.[9] Research on ALL in children conducted by Ffrench and his colleagues[4] showed the percentage of males 67% while females 33%.[4]

The mean age of patients with ALL in children was 7.33 with a standard deviation of 3.94. The youngest

Table 1. Characteristics of pediatric acute lymphoblastic leukemia research subjects

Characteristics of research subjects	amount	%	Mean	SD
Sex				
Boy	9	75		
Girl	3	25		
Age			7.33	3.94
2 years	1	8.3		
3 years	2	16.7		
4 years	1	8.3		
6 years	2	16.7		
7 years	1	8.3		
9 years	1	8.3		
12 years	4	33.3		
Hb before chemotherapy induction phase			9.31	2.65
Hb after chemotherapy induction phase			11.77	1.81
Leukocytes before chemotherapy induction phase			76.90	167.51
Leukocytes after chemotherapy induction phase			8.90	15.05
Platelets before chemotherapy induction phase			70.39	43.20
Platelets after chemotherapy induction phase			214.53	138.79

was 2 years old, while the oldest was 12 years of age, the most were aged 12, four (33.3%) children. This was in contrast with the literature that stated that the highest age group of children was two to four years.[9] This difference may be due to the number of research subjects.

The mean hemoglobin levels of pediatric ALL research subjects before chemotherapy induction phase was 9.31 g/dL. After chemotherapy induction phase, the mean hemoglobin levels became 11.77 g/dL. The mean leukocytes of pediatric Acute Lymphoblastic Leukemia research subjects before chemotherapy induction phase was 76.9×10^3/uL. After chemotherapy induction phase, the mean leukocytes became 8.9×10^3/uL. The mean platelets of pediatric ALL research subjects before chemotherapy induction phase was 70.39×10^3/uL. After chemotherapy induction phase, the mean platelets became 214.53×10^3/uL. Conditions of anemia and thrombocytopenia in the state before chemotherapy induction phase can be caused by hematopoietic cell suppression by leukemia cells accumulating in the bone marrow. This will lead to anemia, thrombocytopenia in the bone marrow and peripheral blood, whereas leukocytes dominated by lymphoblast cells will increase. Acute lymphoblastic leukemia circumstance will occur as a proliferative disorder causing uncontrolled proliferation while apoptotic function was impaired.[9] This situation was different when seen after administration of chemotherapy induction phase and it turned out that in this study all patients were remission.

Wilcoxon Signed Ranked Test Results of lymphoblast proliferation of pediatric ALL in peripheral blood before and after chemotherapy induction phase were as follows:

Table 2. Wilcoxon Signed Rank Test Analysis lymphoblast proliferation of pediatric acute lymphoblastic leukemia in peripheral blood as research subjects before and after chemotherapy induction phase

Lymphoblast proliferation (%)	Mean	SD	Value p (1-tailed)
Before chemotherapy induction phase	7.84	7.75	0.0075
After chemotherapy induction phase	3.20	1.89	

The mean patient lymphoblast proliferation of pediatric ALL research subjects in peripheral blood before chemotherapy induction phase was 7.84%, with a standard deviation of 7.75. After chemotherapy induction phase, the mean patient lymphoblast proliferation of pediatric ALL as research subjects fell to 3.2%, with a standard deviation of 1.89. Wilcoxon Signed Rank Test resulted in a p value of $0.0075<0.05$ so it could be concluded as a significant difference in lymphoblast proliferation of pediatric ALL research subjects in peripheral blood before and after chemotherapy induction phase.

Results of paired samples t test of apoptosis pediatric ALL as research subjects before and after chemotherapy induction phase were as follows:

Table 3. Paired t test samples lymphoblast apoptosis of pediatric acute lymphoblastic leukemia in peripheral blood as research subjects before and after chemotherapy induction phase

Apoptosis (%)	Mean	SD	Value p
Before chemotherapy induction phase	11.50	8.60	0.601
After chemotherapy induction phase	13.42	8.10	

The mean patient lymphoblast apoptosis of pediatric ALL as research subjects in peripheral blood before chemotherapy induction phase was 11.5% with a standard deviation of 8.6. After chemotherapy induction phase, the mean patient lymphoblast apoptosis pediatric ALL as research subjects in peripheral blood was 13.42% with a standard deviation of 8.1. Paired t test samples resulting in a p value of $0.3005>0.05$ concluded that there were no significant differences in lymphoblast apoptosis pediatric ALL as research subjects in peripheral blood before and after chemotherapy induction phase. This study using data from another patient group of examination lymphoblast apoptotic of bone marrow aspiration showed that the mean of lymphoblast apoptotic in bone marrow before chemotherapy induction phase was 10.11% and after was 30.11%, the results revealed a significant increase of lymphoblast apoptotic with a p value 0.200.

Chemotherapy treatment in induction phase by using a class of glucocorticoids such as prednisone or dexamethasone could give meaningful antileukemic effects. Effects on lymphoid cells induced apoptosis and resistance of the cell cycle. Kofler[10] insisted that the ongoing repression of the metabolic pathways through the glucocorticoid receptor regulation contributed to the inhibition of cell cycle.[10] Methotrexate, which is one example of a series of induction therapy has the effect of competition in inhibiting dihydrofolate reductase, which is an enzyme participating in the synthesis of tetrahydrofolate in producing folic acid. Folic acid is required for DNA synthesis, thus causing inhibition to methotrexate therapy DNA.[11] Vincristine of the same dose will inhibit increase of cell proliferation

at 24 hours, 48 hours and 72 hours with levels of 0.113±0.012, 0.078±0.009 and 0.051±0.008 μM.[12] Use of daunorubicin caused restraint DNA repair through topoisomerase II inhibitor that produces barriers of DNA synthesis and RNA.[13] Research conducted by Sur and his colleagues demonstrated that asparaginase inhibited proliferation of leukemic cells by showing the loss of S phase of the cycle cell.[14]

Effects of chemotherapy can impact lymphoblast cell necrosis or premature apoptotic examination, which caused not be detected as apoptotic bodies in this study. A research conducted by Liu[15] proved their lymphoblast apoptosis increase of about <1% to 38% in peripheral blood of patients with pediatric acute lymphoblastic leukemia monitored after 24 hours of administration of chemotherapy compared to apoptosis induction phase starting about 3% to 29%.[15] A Study conducted by Laane[16] stated that dexamethasone induced apoptosis was associated with the activation of Bax (p=0.045) and the down-regulation of Bcl-2 (p=0.016) and or Bcl-xL (p=0.004) in cell cultures of pediatric ALL, as well as showing an increase in apoptosis after administration for 24 hours, 36 hours and 48 hours.[16]

CONCLUSIONS AND SUGGESTIONS

Conclusions drawn based on the results and discussion of this research are as follows: There was a significant decrease in the percentage of lymphoblast proliferation in peripheral blood of pediatric ALL after chemotherapy induction phase; There was no significant increase in lymphoblast apoptosis in peripheral blood of pediatric ALL before and after chemotherapy induction phase; The percentage of lymphoblast proliferation in peripheral blood can be used as an alternative to monitor the success of therapy in pediatric ALL.

Based on the above results, it is recommended: Long-term observation should be made to complete the management of chemotherapy; There should be an uniformity span of the specimen at the time prior to completion of chemotherapy induction phase; A specific marker of lymphocytes examination (examination CD 3, CD19) for separating the monocyte series is clearly needed.

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