Original Article

Received: 2021-03-07
Accepted: 2021-07-29

BCCR
2021, No 1, Vol 13 :55-62
www.bccrjournal.com

The Effect of Ursodeoxycholic acid and N-acetyl cysteine on Lymphoblast Viability

Ali Aycicek[1*], Tuba Nur Tahtakesen[1], Cengiz Bayram[1]

1 Health Sciences University, BasaksehirCam and Sakura City Hospital, Pediadric Hematology/ Oncolog Clinic, 34480, Istanbul, TURKEY.
*Corresponding author: Ali Aycicek, Istanbul Health Sciences University, Basaksehir Cam and Sakura City Hospital, Pediadric Hematology/ Oncolog Clinic, Istanbul, TURKEY.
E-Mail:ayciceka@hotmail.com
Number of tables: 2
Number of figures: 5
Word count:3446
Number of color figures: 0
Number of text pages:8
Number of reference pages: 2
Key words: children, acute lymphoblastic leukemia, ursodeoxy cholic acid, N-acetyl cystein
Address of Corresponding Author
Ali AYCICEK, M.D.
Basaksehir Cam and Sakura City Hospital
Pediadric Hematology/Oncolog Clinic
34480,Istanbul, TURKEY.

Fax: +90 212 512 20 86
Telephone : +90 212 909 60 00
E-Mail:ayciceka@hotmail.com

ABSTRACT

Aim: To investigate invitro ursodeoxycholic acid (UDCA) and N-acetyl cysteine (NAC) effect on blast cell viability in children newly diagnosed with acute lymphoblastic leukemia (ALL).

Patients and Methods: Samples were obtained from 52 newly diagnosed ALL patients aged 1 to 17 years. UDCA and NAC were added at clinically relevant concentrations (0-300 micrograms) onto 5x10^5 cells treated at room temperature in a dark place. Untreated and treated cells were stained with 7-amino-actinomycin D (7AAD PE) and analyzed by flow cytometry.

Results: Median (interquartile range; IQR) blast percentage and incubation time were 90% (11) and 18 (1.5) hours, respectively. The dead/live blast cells ratio (7AAD+) was lower in lymphoblasts treated with all NAC concentrations than untreated controls ($P < 0.001$). The use of NAC was noted to, regardless of concentration, contribute to lymphoblasts viability. On the contrary, the dead/live blast cells ratio in samples treated with UDCA at the abovementioned concentrations was relatively high, suggesting the protective role for both hepatotoxicities and against leukemia. However, the difference was not statistically significant ($P > 0.05$). There was also no correlation between different doses of UCDA and NAC regarding blast cell viability ($P > 0.232$).

Conclusion: The present study showed that in vitro NAC use had a protective effect on lymphoblast viability in newly diagnosed ALL patients before starting chemotherapy. Patient-derived ALL cells can be successfully analyzed ex vivo in a short and different period without loss of blasts.

Keywords: Acute Lymphoblastic Leukemia, Children, N-acetyl cysteine, Ursodeoxycholic acid

INTRODUCTION:

All anticancer drugs can cause hepatotoxicity that may result in elevated liver enzyme levels and parenchymal cell injury (1-3). The reactions are generally dose-independent and idiosyncratic due to immunologic mechanisms. Less common are dose-dependent, predictable toxic effects of a medication or its metabolites. Chemotherapeutic agents, alone or in combination, may cause hypersensitivity reactions or direct hepatic toxicity, and altered liver function may alter drug metabolism and cause an increased risk of nonhepatic toxicity. In cancer chemotherapy, however, dosing decisions are often made based on limiting toxicity (4). Liver injury during cancer chemotherapy leads to interruption, dose reduction, or delay in cancer treatment, resulting in treatment failure and ultimately leading to cancer recurrence and poor survival. Management options in clinical practice for hepatic toxicity related to cancer treatment include discontinuing the suspected causative agent and using corticosteroids and ursodeoxycholic acid (UDCA)(5). N-acetyl cysteine (NAC) has also been shown to be effective as antioxidant adjuvant therapy in children with ALL to reduce chemo-/radiotherapy-related toxicities during the initial period of treatment (6). Despite the use of NAC and/or UDCA for hepatotoxicity development during cancer treatment, it is not known if there are any effects of these agents on blast cells.

Functional studies with primary blasts from children with ALL cannot be widely used due to the difficulty of expanding ALL cells in vitro. ALL blasts are highly dependent on their in vivo environment and rapidly undergo apoptosis ex vivo. Short-term in vitro experiments have been developed to test drug sensitivity; however, their use has not been widely practiced as ALL cells in these assays are rapidly reduced, even they are not exposed to any chemotherapeutic compounds (7).

The present study aimed to evaluate in vitro effects of NAC and UDCA on lymphoblasts and determine if there were any protective or apoptotic effects on leukemic cells in newly diagnosed ALL before starting chemotherapy.

Material and methods

This study included 52 children with newly diagnosed ALLbetween December 2018 and April 2020.Patients with ambiguous lineage ALL and patients who received glucocorticosteroids and/or other antileukemic agents were excluded from the study (8). Peripheral blood or bone marrow aspirate samples were collected into tubes without EDTA, and then were smeared on slides and stained with Wright-Giemsa stain, and whole blood analyses were performed with SYSMEX XE-2100. A pediatric hematologist made morphological identification of blast cells, and the percentage of lymphoblasts was recorded. The flow cytometry protocol was used to detect blasts, as described in detail previously (9). Cell acquisition was implemented with FacsCantoII (B.D. Biosciences) using FacsDiva V8 (B.D. Biosciences) for data analysis. Bone marrow aspiration material or whole blood sample belonging to each patient diagnosed with acute lymphoblastic leukemia in the flow cytometry laboratory were included in this study without waiting, and the experiments were started by adding UDCA and NAC doses as follows. If the sample had to be kept waiting, it was kept in the dark at room temperature (9).The stock solution was prepared at a concentration of 100 µg/3 µL and 30 µg/3 µL for UDCA and NAC study samples, respectively. And then, 0, 100, 200, 300 µgof UCDA and 0, 30, 60, 90 µgof NAC were added onto 5x10^5 cells treated for different periods (6-18 hours) at room temperature in a dark place (10).The control group, defined as NAC or UDCA untreated samples, were incubated in the same place without the addition of NAC and UDCA. Untreated and treated cells were stained with 7AAD P.E. (Becton-Dickinson, San Jose, CA, USA) according to the manufacturer's instructions for detection of apoptosis, incubated at room temperature for 15 min before flow cytometry analysis (FACSCantoII, Becton-Dickinson, San Jose, CA, USA) with an excitation wavelength of 488 nm and a maximum emission wavelength of 677 nm. Cell acquisition was conducted, collecting per tube 30.000 events using the FacsDiva V8 (B.D. Biosciences) software for data analysis. The panel of combinations was limited to per tube of 7AAD/CD45. Viable cells (no

staining), apoptotic cells, and late-apoptotic cells were detected and quantified as a percentage of the entire cell and alive population (Figure 1, 2). The sum of apoptotic and late apoptotic cells was considered as cell death population (11). Low side scatters, moderate and bright 7AAD leukemic cells were designated as P1 gate. Absolute dead blast values were computed using the cell count of the sample (assessed with an FCM) and the relative dead estimate from the FCM analysis. Dead/cell, dead/alive, and p1 / dead cell ratios were calculated for each tube's mentioned amounts of drugs (Figure 1,2). A bivariate dot plot identified leukemic cells and normal B-lymphocytes cells as CD45 negative-dim positive cells with low side scatter. The device set-up was optimized by analyzing Calibrite beads (B.D. Biosciences) and normal peripheral B cells stained with CD3/16+56/CD45 (12). This study was approved by the institutional ethical committee (Health Sciences University, Istanbul Kanuni Sultan Suleyman Education and Research Hospital Ethical Committee; approval number: 2019/39). It was carried out according to informed consent guidelines.

Statistical analysis

Normality of variances was accomplished using the Kolmogorov-Smirnov test. The data were expressed as median (interquartile range) and were compared using the Mann-Whitney U, Friedman, Wilcoxon signed-rank test. Bivariate associations between variables were assessed with Pearson's correlation test. The differences were considered statistically significant at $P < 0.05$. Statistical analyses were conducted using the IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

RESULTS:

There were 29 (56%) male and 23 female (44%) patients, and the median age of patients at diagnosis was 7.6 years (range 1-18 years). According to the ALLIC BFM risk stratification criteria, 3 (6%) children were classified

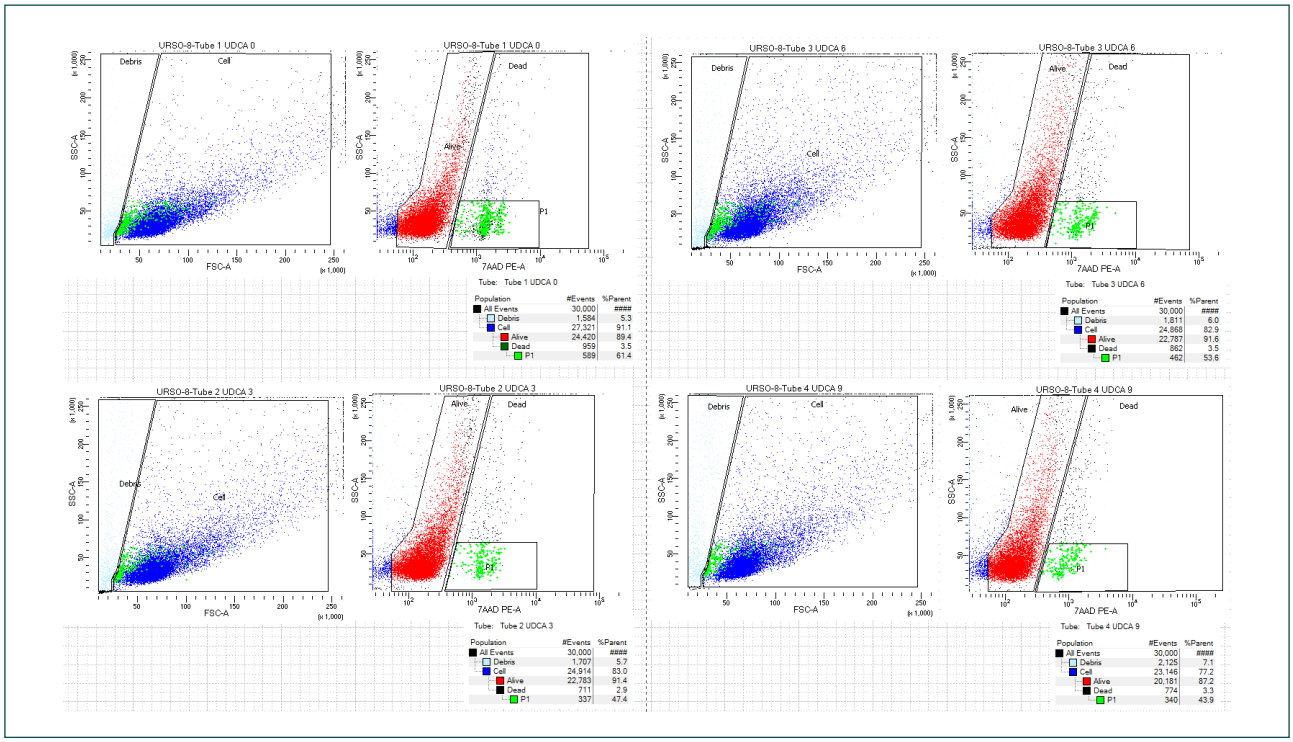

Figure.1. The effect of different UDCA concentration on the viability of lymphoblasts of case 6.
Gating strategy to identify total cell, alive, dead and leukemic dead cells (P1)inuntreated (tube 1, upper left), and treated with 100 μg (lower left), 200 μg (upper right), and 300 μg (lower right) of UDCA for 18 hours.;Total cell were all cells except debris, alive were not stained with 7-AAD, apoptotic and late apopitotic blast cells were low SSC moderate and bright 7AAD that were designated P1.FSC forward scatter, SSC side scatter, NAC, N-acetyl cystein, UDCA, ursodeoxycholic acid.

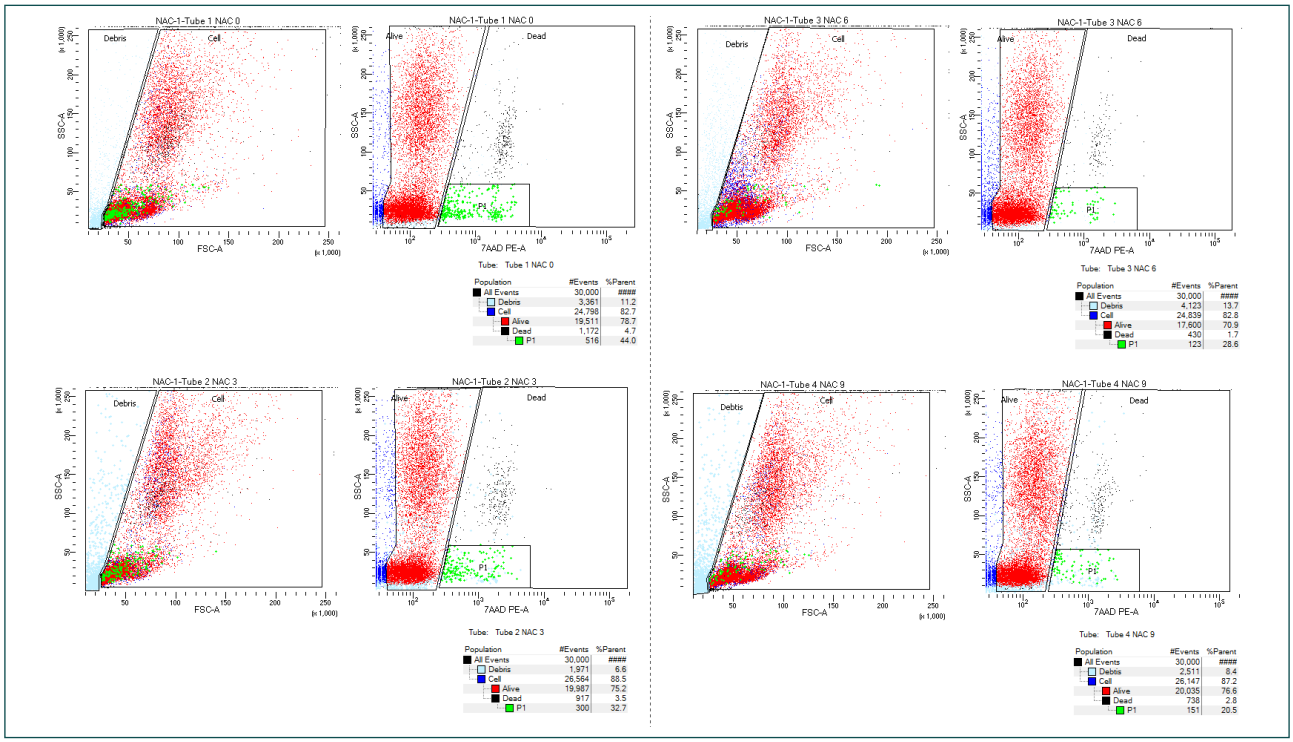

Figure.2. The effect of different NAC concentration on the viability of lymphoblasts of case 1.
Total cell, alive, dead and leukemic dead cells (P1) inuntreated (tube 1, upper left), and treated with 30 µg (lower left), 60 µg (upper right), and 90 µg (lower right) of NAC for 18 hours.

Age, median, IQR	**7.6 (8.4)**
Gender, n, %	
Male	**29 (56%)**
Female	**23 (44%)**
Blast percentage of samples, median, IQR	**90 (11)**
Blast phenotype, n, %	
Pre-B cell	**45 (86%)**
T cell	**7 (14%)**
Risk status, n, %	
Standard	**3 (6%)**
Intermediate	**38 (73%)**
High	**11 (21%)**
The incubation time of samples, hour, IQR*	**18 (1.5)**

*interquartile range; P1: dead blast; NAC: N_acetyl cystein;UDCA: ursodeoxycholic acid.
Table 1. Patients characteristics.

as standard risk, 38 (73%) were intermediate risk, and 11 (21%) were high risk (Table 1). Forty-five (86%) children had a pre-B cell phenotype, 7 (14%) had a T cell phenotype. Median (IQR) white blood cell count, blast percentage,and incubation time for the studied samples were 70.000 (180.000)/mm3, 90% (11), and 18 (1.5) hours, respectively.

As shown in Table 2 and Figures 3-5, dead/alive, dead/cell, p1/dead ratios (7AAD+) of blasts after median 18 (1.5) hours incubation with 30 µg NAC concentration were lower than untreated controls ($p < 0.001$) (Figure 3-5). Similar results were obtained at 60 and 90 µg concentrations of NAC, suggesting a dose-independent effect. On the contrary, UDCA in the present concentrations relatively increased dead cell ratios (pro-oxidant) rather than contributing to the viability of the cells. However, the difference was not statistically significant ($P > 0.05$). There was also no correlation between different doses of UCDA and NAC regarding blast cell viability ($P > 0.232$). Results of apoptosis tests are summarized

UDCA, median, IQR	Untreated	100µg, 3 µL	200 µg, 6 µL	300 µg, 9 µL	P
Dead/Total cell	6.8 (15)	9.4 (15)	7.9 (13)	14) 9.7)	0.533
Dead/Alive cell	7.5 (22)	13 (21)	11 (17)	15 (21)	0.429
P1/Dead	68 (26)*	64 (34)	59 (28)	59 (28)	<0.001
NAC, median, IQR	**Untreated**	**30 µg (3 µL),**	**60 µg(6 µL),**	**900 µg (9 µL),**	**0.296**
Dead/Total cell	8.3 (23)*	4.6 (12)	5.1 (12)	5.6 (17)	<0.001
Dead/Alive cell	9.8 (37)*	6.2 (17)	6.8 (18)	7.5 (24)	<0.001
P1/Dead	73 (32)*	64 (22)	61 (33)	61 (31)	<0.001

*The different group
UDCA: Ursodeoxycholic acid; NAC: N-acetyl cystein; IQR: interquartile range,

Table 2. Median apoptotic cell rate in study samples untreated and treated with NAC and UDCA assessed by 7AAD.

in Table 2. There was no correlation between incubation time and apoptotic cell ratios ($P > 0.05$). Untreated samples incubated as without NAC or UDCA; dead defines apoptotic cells; P1, low side scatter moderate and bright 7AAD blastic cells.

DISCUSSION

This paper showed that patient-derived ALL cells could be successfully analyzed ex vivo in a short and different time period without loss of blasts. The detection of cell viability is critical when evaluating the physiological state of cells, such as in response to cytotoxic drugs or during the progression of neoplasia and other disease states(13). Dye exclusion is a method to identify the two cell populations. Live cells have intact membranes that exclude a variety of dyes that easily penetrate the disrupt, permeable membranes of apoptotic or non-viable cells. Flow cytometry provides a simple, rapid, cheap, reliable method to quantify viable cells in a cell suspension (13). 7-AAD is animpermeant membrane dye that assay for quantification of apoptosis with flowcytometry in peripheral blood mononuclear cells (PBMCs) and human leukemia cells (11),(14), (15). In this study, we showed that determination of cell viability with 7-AAD is critical when evaluating the physiological state of cells, such as in response to antioxidant drugs in childhood ALL.

Some cancer patients or their parents use antioxidants as dietary supplements to alleviate chemotherapy-induced toxicitiesto increase long-term survival. However, little is known about the effectiveness and safety of antioxidant use during chemotherapy, including its interaction with chemotherapy agents(16). There is great concern about antioxidant use during cancer therapy, which may increase the viability or proliferation capacity of leukemia cells (17). Hepatic toxicity is one of the most commonly encountered side effects of childhood cancer therapy.UDCA and NAC are widely used agents in chemotherapy-related hepatic toxicity for the purpose mentioned above. Methotrexate, one of the backbone chemotherapeutic agents in childhood ALL treatment, which can induce apoptosis in hepatocytes by increasing tumor protein 53 (TP53) levels, also causes oxidative tissue injury in the liver by increasing reactive oxygen metabolites levels and lipid peroxidation. These mechanisms could be inhibited by using UDCA and NAC (6), (18). In an experimental study, Uraz et al. showed that

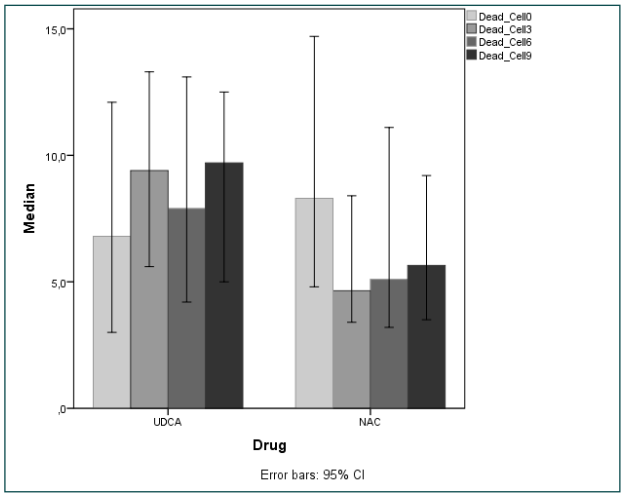

Figure.3. Median dead/total cell ratio from 52 samples. Untreated (dead_alive0), treated with 100 µg (dead_alive3), 200 µg (dead_alive6), and 300 µg (dead_alive9) of UDCA and untreated (dead_alive0), treated with 30 µg (dead_alive3), 60 µg (dead_alive6), and 90 µg (dead_cell9) of NAC, respectively.. NAC, N-acetyl cystein, UDCA, ursodeoxycholic acid.

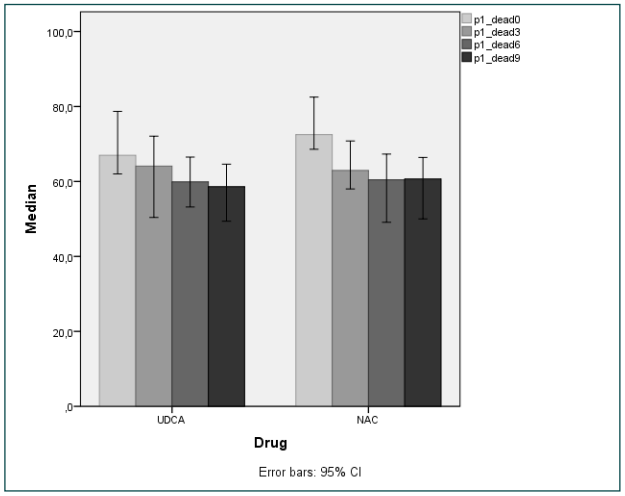

Figure.5. Median P1/dead cell ratio from 52 samples. Untreated (dead_cell0), treated with 100 µg (dead_cell3), 200 µg (dead_cell6), and 300 µg (dead_cell9) of UDCA and untreated (dead_cell0), treated with 30 µg (dead_cell3), 60 µg (dead_cell6), and 90 µg (dead_cell9) of NAC, respectively

usinga single dose of 20 mg/kg methotrexate for 6 days caused hepatic and/or cholestatic toxicity and hepatocyte necrosis in histopathological examination of liver tissue. By contrast, normal liver histology was observed when methotrexate was given with UDCA at 50 mg/kg. They concluded that methotrexate-induced liver parenchymal cell injury and hepatocyte necrosis could be

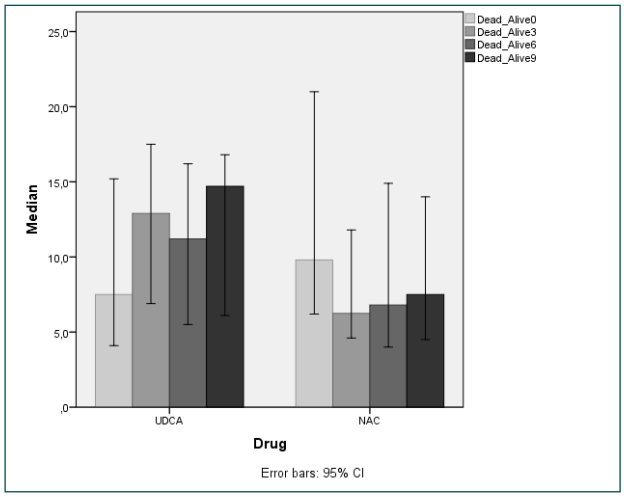

Figure.4. Median dead/alive cell ratio from 52 samples. Untreated (P1-dead0), treated with 100 µg (P1_dead3), 200 µg (P1_dead6), and 300 µg (P1_dead9) of UDCA and untreated (P1_dead0), treated with 30 µg (P1_dead3), 60 µg (P1_dead6), and 90 µg (P1_dead9) of NAC, respectively. NAC, N-acetyl cystein, UDCA, ursodeoxycholic acid.

prevented by the hepatoprotective effect of UDCA (18). However, the latter study is far from clinical use in the context of methotrexate use in childhood cancer treatment, as methotrexate is given as 36-hour or usually, 24-hour continuous infusion in the consolidation phase of treatment, while at a dose of 20-30 mg/m2 once a week in ALL maintenance therapy (19). In this regard, Bordbar et al. investigated hepatoprotective effects of UDCA in pediatric patients with ALL receiving methotrexate in the maintenance phase of treatment. There was no significant difference in patients receiving methotrexate + UDCA in this study compared to patients receiving methotrexate alone (16). In the current study, the ratio of dead/alive cells in patients treated with UDCA, although statistically not significant, was high, suggesting the protective role of UDCA for both hepatotoxicities and against leukemia. This is an in vitro study and done in ALL patients before chemotherapy. Clinical studies are needed to confirm the present study's results. Another endpoint of our study was to address whether NAC's protection effect on leukemic blast cells is due to its negative effect on induced-ROS production, which protects the liver and body from oxidative damage. The increase in basal ROS levels renders cancer cells highly depend-

ent on antioxidant systems and therefore vulnerable to agents that block ROS detoxifying mechanisms(20). Thus, we also assessed the dead cells/alive cells ratio regarding the effect of NAC on lymphoblasts at a different concentration compared to untreated samples without NAC. The ratio of dead cells/ alive cells was lower than untreated patients, suggesting NAC's protective effects on lymphoblasts, an undesirable effect that may lead to poor prognosis.

The pretreatment bone marrow blast burden in pediatric patients with ALL is reported to be 3x10^9. It is accepted to decrease 3 logs after the cancer cell induction treatment, and the decrease in similar rates continues in the following treatment cycles(21). Despite achieving morphological and molecular remission even in maintenance, a significant number of cells may be present in the body responsible for subsequent relapse (22). Therefore, factors leading to treatment delay have prognostic significance for achieving a good response to chemotherapy and long-term survival. Thus, elevated liver function tests, one of the most common reasons for the chemotherapy interruption, should not indicate treatment adaptation and/or the initiation of antioxidant agents such as NAC and UDCA if liver function is normal. (2).

There are some limitations to this study. It was an in vitro study, samples were studied in a dark place at room temperature, and drug doses were determined by considering therapeutic doses and the amount ofbody fluid composition. However, the current study is the first to demonstrate the protective effect of NAC use on lymphoblast viability that may cause ALL treatment failure and poor survival. Also, patient-derived ALL cells can be successfully analyzed ex vivo within a short period of time without loss of blasts.

Author contributions

BA.A. designed the study, developed the flow cytometry assays, performed the statistical analysis, analyzed the data, and wrote the manuscript. T. N. T. performed flow cytometry assay and data acquisition; C. B. revised and edited the article.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest concerning this article's research, authorship, and/or publication.

Funding

The author(s) received no financial support for this article's research, authorship, and/or publication.

ORCID iD

Ali Aycicek: https:/orcid.org/ 0000-0001-8951-4750

REFERENCES

1. Floyd J, Mirza I, Sachs B, et al. Hepatotoxicity of chemotherapy. Semin Oncol 2006;33:50–67.
2. Ebbesen MS, Nygaard U, Rosthøj S, et al. Hepatotoxicity During Maintenance Therapy and Prognosis in Children With Acute Lymphoblastic Leukemia. J Pediatr Hematol Oncol 2017; 39:161-166.
3. Okamura A, Nishimura M, Sanada Y, et al. L-Asparaginase-induced fulminating liver dysfunction. Int J Hematol 2013;98:6-7.
4. King PD, Perry MC. Hepatotoxicity of chemotherapy. Oncologist. 2001;6:162–176.
5. Giordano C, Rivas J, Zervos X. An Update on Treatment of Drug-Induced Liver Injury. J Clin Transl Hepatol 2014;2:74-79.
6. Al-Tonbary Y, Al-Haggar M, El-Ashry R, et al. Vitamin e and N-acetylcysteine as antioxidant adjuvant therapy in children with acute lymphoblastic leukemia. Adv Hematol 2009;689639.
7. Pal D, Blair HJ, Elder A, et al. Long-term in vitro maintenance of clonal abundance and leukaemia-initiating potential in acute lymphoblastic leukaemia. Leukemia 2016;30:1691–700.
8. Modvig S, Madsen HO, Siitonen SM, Rosthøj S, Tierens A, Juvonen V, et al. Minimal residual disease quantification by flow cytometry provides reliable risk stratification in T-cell acute lymphoblastic leukemia. Leukemia 2019;33:1324-1336.
9. Dworzak MN, Gaipa G, Ratei R, et al. Standardization of flow cytometric minimal residual disease evaluation in acute lymphoblastic leukemia: Multicentric assessment is feasible. Cytometry B Clin Cytom 2008;74:331-340.
10. Diks AM, Bonroy C, Teodosio C, et al. Impact of blood storage and sample handling on quality of high dimensional flow cytometric data in multicenter clinical research. J Immunol Methods 2019;475:112616.
11. Zembruski NC, Stache V, Haefeli WE, et al. 7-Aminoactinomycin D for apoptosis staining in flow cytometry. Anal Biochem. 2012;429:79-81.
12. Ratei R, Basso G, Dworzak M, et al, Monitoring treatment response of childhood precursor B-cell acute lymphoblastic leukemia in the AIEOP-BFM-ALL 2000 protocol with multiparameter flow cytometry: predictive impact of early blast reduction on the remission status after induction. Leukemia 2009; 23:528-534.
13. Philpott NJ, Turner AJ, Scopes J, et al. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad

spectrum of application compared with other techniques. Blood 1996;87:2244-2251.
14. Stacchini A, Aliberti S, Demurtas A, et al. Flow cytometry identification of nonhemopoietic neoplasms during routine immunophenotyping. Int J Lab Hematol 2019;41:208-217.
15. Ayuk FA, Atassi N, Schuch G, et al. Complement-dependent and complement-independent cytotoxicity of polyclonal antithymocyte globulins in chronic lymphocytic leukemia. Leuk Res 2008;32:1200-1206.
16. Bordbar M, Shakibazad N, Fattahi M, et al. Effect of ursodeoxycholic acid and vitamin E in the prevention of liver injury from methotrexate in pediatric leukemia. Turk J Gastroenterol 2018;29:203-209.
17. Kocyigit A, Guler EM, Karatas E, et al. Dose-dependent proliferative and cytotoxic effects of melatonin on human epidermoid carcinoma and normal skin fibroblast cells. Mutat Res Genet Toxicol Environ Mutagen 2018;829-830:50-60.
18. Uraz S, Tahan V, Aygun C, et al. Role of ursodeoxycholic acid in prevention of methotrexate-induced liver toxicity. Dig Dis Sci 2008;53:1071–1077.
19. Stary J, Zimmermann M, Campbell M, et al. Intensive chemotherapy for childhood acute lymphoblastic leukemia: results of the randomized intercontinental trial ALL IC-BFM 2002. J Clin Oncol 2014;32:174-184.
20. Schoeneberger H, Belz K, Schenk B, et al. Impairment of antioxidant defense via glutathione depletion sensitizes acute lymphoblastic leukemia cells for Smac mimetic-induced cell death.Oncogene 2015;34:4032-4043.
21. Uckun FM1, Stork L, Seibel N, et al. Residual bone marrow leukemic progenitor cell burden after induction chemotherapy in pediatric patients with acute lymphoblastic leukemia. Clin Cancer Res 2000;6:3123-3130.
22. Parikh SK, Uparkar UP. Assessment of minimal residual disease in childhood acute lymphoblastic leukemia. J Appl Hematol 2016;7:47-53.
23. Kong Y, Song Y, Tang FF et al. N-acetyl-L-cysteine improves mesenchymal stem cell function in prolonged isolated thrombocytopenia post-allotransplant. Br J Haematol 2018;180:863-878.
24. Dworzak MN, Fröschl G, Printz D, et al. Prognostic significance and modalities of flow cytometric minimal residual disease detection in childhood acute lymphoblastic leukemia. Blood 2002;99:1952-1958.
25. Kong Y, Shi MM, Zhang YY, et al. N-acetyl-L-cysteine improves bone marrow endothelial progenitor cells in prolonged isolated thrombocytopenia patients post allogeneic hematopoietic stem cell transplantation. Am J Hematol 2018;93:931-942.
26. Al-Rubeai M, Welzenbach K, Lloyd DR, et al. A rapid method for evaluation of cell number and viability by flow cytometry. Cytotechnology 1997;24:161-168.