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Apoptotic Effect of Heparin on the Lymphoblasts Using DNA Analysis and Measurements of Intracellular Calcium

Erol Erduran[1]*, Yusuf Gedik[2], Yavuz Tekelioglu[3], Yuksel Aliyazıcıoglu[4] and Tugba Bayraktar[5]

[1]*Professor, Department of Pediatric Hematology, School of Medicine, Karadeniz Technical University, Trabzon, Turkey*
[2]*Professor, Department of Pediatrics, School of Medicine, Karadeniz Technical University, Trabzon, Turkey*
[3]*Associate Professor, Department of Histology and Embryology, School of Medicine, Karadeniz Technical University, Trabzon, Turkey*
[4]*Associate Professor, Department of Biochemistry, School of Medicine, Karadeniz Technical University, Trabzon, Turkey*
[5]*Assistant Professor, Department of Pediatrics, School of Medicine, Karadeniz Technical University, Trabzon, Turkey*

Abstract

Background: Beside its anti-proliferative, anti-hypertensive and anti-inflammatory effects, heparin has shown the apoptotic effect in lymphoblasts. In the study, it is aimed to show the apoptotic effect of heparin in lymphoblasts with measuring intracellular calcium and using DNA analysis by flow cytometry, *in vitro*.

Methods: Twenty-three newly diagnosed acute lymphoblastic leukemia patients were included in the study. We added 10 and 20 U/ml heparin into the seperated lymphoblast samples and determined the percentages of apoptosis and intracellular Ca^{++} levels at 0, 1 and 2 hours by flow cytometry, *in vitro*.

Results: The apoptotic effect on the lymphoblasts were established in 10 and 20 U/ml heparin concentrations at 0, 1 and 2 hours (p=0.005). The apoptotic effect of heparin in lymphoblasts was higher at the first hour than those at 0 and 2 hours in 10 and 20 U/ml heparin concentrations (p=0.005). The highest apoptosis was determined in 20 U/ml heparin concentration at the first hour. Statistically significant increase in intracellular Ca^{++} levels were determined in 10 and 20 U/ml heparin concentrations at 1 and 2 hours (p=0.005). In 10 and 20 U/ml heparin concentrations, intracellular Ca^{++} levels were significantly higher at the first hour than 0 and 2 hours (p=0.005). The highest intracellular Ca^{++} concentration was determined in 20 U/ml heparin concentration at the first hour.

Conclusion: Heparin induces apoptosis in lymphoblasts and intracellular Ca^{++} levels of the lymphoblasts synchronously increase with apoptosis. The increase of intracellular Ca^{++} level supports a concept that the mitochondria plays a role heparin-induced apoptosis in lymphoblasts.

Keywords: Apoptosis; Acute lymphoblastic leukemia; Heparim; Calcium

Abbreviations: Cyt C: Cytochrome C; ALL: Acute Lymphoblastic Leukemia; BMA: Bone Marrow Aspirations; EDTA: Ethylendiaminetetraacetate; PI: Propidium Iodide; FCM: Flow Cytometry

Introduction

Beside its anticoagulant, anti-inflammatory, antihypertensive and antiproliferative effects [1-3], heparin has the apoptotic effect in lymphoblasts, *in vitro* [4-6]. Manaster et al. [7] claimed that 50, 100 and 200 U/ml concentrations of heparin induce apoptosis in human peripheral blood neutrophils, *in vitro*. In our studies, lymphoblasts were incubated with 0, 5, 10 and 20 U/ml concentrations of heparin at 0, 1 and 2 hours and the highest apoptosis was determined in 20 U/ml heparin concentration at first hour [4-6]. The expression of fas, the activities of caspase-3 and -8 increased and the expression of bcl-2 decreased when lymphoblasts were incubated with heparin in 10 and 20 U/ml concentrations at 1 and 2 hours. We first reported the apoptotic effect of heparin in lymphoblasts and stated that heparin induces apoptosis via the activation of the extrinsic pathway [5,6]. Recently, we claimed that heparin induces intrinsic apoptosis of lymphoblasts by the activation of caspase-9 and Cytochrome C (Cyt C) [8]. The cytosolic Ca^{++} levels increase during the activation of the intrinsic apoptotic pathway. The increase of the intracellular Ca^{++} initiates the mitochondria-mediated apoptosis and causes the release of Cyt C from the mitochondria. Cyt C binds Apaf-1 (apoptosis protease activating factor-1) and ATP binds both Cyt C and Apaf-1. The inactive caspase-9 and -3 transform into their activate forms and consequently, apoptosis develops [9-11]. In the study, we aimed to measure the intracellular Ca^{++} levels in lymphoblasts incubated with heparin and to indicate mitochondria-mediated apoptosis of heparin by flow cytometry (FCM).

Subjects and Methods

The bone marrow samples of 32 newly diagnosed children with acute lymphoblastic leukemia (ALL), aged between 2-11 years (14 boys and 9 girls), were included in the study. The approval of local ethic committee and the written parental consent were received. Bone marrow aspiration (BMA) was done for definitive diagnosis. The BMA samples were put into the tubes with ethylendiaminetetraacetate (EDTA). The BMA samples with EDTA were diluted with the same amount of phosphate buffer saline (PBS: 0.8 g NaCl, 0.144 g Na_2HPO_4,

***Corresponding author:** Dr. Erol Erduran, Professor, Department of Pediatric Hematology, School of Medicine, Karadeniz Technical University, Trabzon, Turkey, Tel: 0090 462 377 54 80; Fax: 0090 462 325 05 18/0090 532 468 62 32; E-mail: erolerduran@yahoo.com

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0.02 g KCl, 0.02 g KH_2PO_4, 80 ml water) in the tubes and the samples were put onto 3 ml ficoll-hypaque 1007 solution (Lymphocyte separation medium- Cat No: 110K6006 Sigma Diagnostic INC. St Louis, MO 63178, USA). The samples were centrifuged at 700 g. for 30 minutes at room temperature. The upper layer of the sample after centrifugation, which contained the mononuclear cells (lymphoblasts), was taken with a disposable pipette, washed with PBS for two times for removing Ficoll, put into RPMI 1640 solution (041-01875 M Gibco, Grand Island, N.Y. 14072, USA) and stored at -80°C until the study. The samples were then thawed at room temperature. The viability of lymphoblasts was investigated by using acridin orange ethidium bromide under immunofluorescence microscope and the samples whose lymphocyte viabilities above 90 percent were included in the study. Blasts in each sample were suspended at a concentration of 1-2 x 10^5 cells/mL in RPMI 1640 with L-glutamine without sodium bicarbonate medium (sigma R-6504). Nine samples were excluded from the study because their lymphocyte viabilities were lower than 90% and 23 samples whose lymphocyte viabilities were higher than 90% were included in the study. The typing of the cells in the samples were done at diagnosis before storing at -80°C by using the monoclonal antibodies such as CD_3 PE (Coulter PN IM1282), CD_4 FITC (Coulter PN IM1107), CD_5 FITC (Coulter PN IM1291), CD_7 FITC (Coulter PN IM0585), CD_8 FITC (Coulter PN IM1264), CD_{10} FITC (Coulter PN IM0471), CD_{13} FITC (Coulter PN IM0778), CD_{14} PE (Coulter PN IM0650), CD_{19} FITC (Coulter PN IM1284), CD_{20} FITC (Coulter PN IM1455), CD_{22} FITC (Coulter PN IM1207), CD_{33} PN (Coulter PN IM1179), CD_{34} FITC (Coulter PN IM1247), CD_{45} FITC (Coulter PN IM0782), CD_{56}, HLA-DR (Coulter Company PN IM 1255), MPO FITC (Coulter PN IM1874) and the apoptotic index was determined by FCM (Epics Elite-Miami, USA). The FCM includes excitation source and the emission filters. The samples were labeled with double monoclonal antibodies and studied after gating with the control monoclonal antibodies. Forward scatter (FCS) and side scatter (SSC) were used for discarding debris. The monoclonal antibodies higher than 20 percent were accepted as positive.

All patients had B cell ALL according to the results of FCM analysis. Apoptosis was studied after thawing the samples. Blast samples of 100 µl were put into 3 tubes and completed to 1 ml with PBS in each tube, final cell concentration in each tube was 1-3 x 10^6 cells/ml. The pure heparin (Sigma H 3149, St Louis, MO 63178, USA) prepared at 0 (PBS), 10 and 20 U/ml concentrations were added into each tube. These samples were treated with Beckman Coulter DNA Prep Reagent (PN 66007055, Miami- Florida, USA) and the membranes of the blasts were pored with DNA prep LPR solution. DNAs and RNAs of the blasts were marked with propidium iodide (PI). The band pass filter was used to measure PI. The marked RNAs were removed with ribonuclease and the marked DNAs remained in the medium. The cell groups were determined with using the photomultiplier-4 (PMT-4) peak on the X axis and the PMT-4 integral on Y axis by FCM after incubation with PI for 15 minutes. Therefore the doubled cells were eliminated and the graphics were obtained in the form of the PMT-4 integral on X axis and the cell count on Y axis. 0 hour was accepted as a time that the heparin was just added on the blast cells. Apoptosis was determined at 0, 10 and 20 U/ml heparin concentrations and at 0, 1 and 2 hours by FCM. Multicycle analyse program was used to detect of apoptosis. The blast cells ongoing to apoptosis were dyed with PI less than the non-apoptotic cells and showed hypodiploid sub-G1 peak on the DNA histogram [4-7,12,13].

Determining Intracellular Ca^{+2} Levels

The intracellular Ca^{++} levels were studied after thawing the samples at the same time with apoptosis and the viability of lymphoblasts. After thawing the samples in room temperature, 20 µl BD indo 1 (Cat No: 343214 (FITC) Becton Dickinson Biosciences Product Immunocytometry, San Diego, USA) was added onto the 100 µl blast samples incubated with 0, 10 and 20 U/ml heparin concentrations and treated with Beckmancoulter Immuneprep Reagent System (Cat No: PN 7507950, Miami-Florida, USA). The intracellular Ca^{++} levels were determined 0, 1 and 2 hours by FCM as percent. Ultraviolet lase line (351-363 nm) -100-200 mv was used to collect the fluorescence. Light scatter and forward standard set up with removal of the blocking filter in the FALS detector. 1-2 x 10^5 cells/mL were analysed per sample as mentioned previously. The gating strategy was FCS and SSC.

Statistics

In the study, the data was supporting the non-parametric conditions. The compatibility of the data to normal distribution was investigated by Smirnov test. Annova tests (Bonferroni test as post hoc) were used in the data concordance with normal distribution and Kruskal Wallis test (Mann Whitney U test as post hoc) was used in the data not in concordance with normal distribution when the measurable data of three groups were compared. Variance analysis in the repetitive measurements (Paired t test as post hoc) was used in the data concordance with normal distribution and Friedman test (Wilcoxon test as post hoc) was used in the data not in concordance with normal distribution in the comparison of the repetitive measurements within groups. Pearson Correlation Analysis was used in the data concordance with normal distribution and Spearman Correlation Analysis was used in the data not concordance with normal distribution to determine the relation between the increase of intracellular calcium and the percentages of apoptosis.

The results were calculated as arithmetic mean ± standard deviation (SD).

Results

The percentages of apoptosis of the blast cells in 0, 10 and 20 U/ml heparin concentrations at 0, 1 and 2 hours are shown in Table 1. As shown in Table 1, apoptosis was not determined 0 U/ml heparin levels at 0, 1 and 2 hours. There were statistically significant differences between the percentages of apoptosis in 10 and 20 U/ml heparin concentrations at 0, 1 and 2 hours (p=0.005) and the percentages of apoptosis in 10 and 20 U/ml heparin levels at first hour were higher than those at 0 and 2 hours (p=0.005). The percentages of apoptosis in 20 U/ml heparin concentration at 0, 1 and 2 hours were significantly

Heparin Time (hour)	0 U/ml (X ± SD) (min-max)	10 U/ml (X ± S.D) (min-max)	20 U/ml (X ± S.D) (min-max)
0	0	10.09 ± 1.27[a] 8.82 - 11.36	12.76 ± 1.08[d] 10.60 - 13.84
1	0	18.81 ± 1.19[b] 17.62 - 20.0	21.52 ± 1,25[e] 20.27 - 22.77
2	0	13.90 ± 1.06[c] 12.84 - 14.96	15.7 ± 1.41[f] 14.29 - 17.11

a-d, a-b, a-c, d-e, d-f, e-f, b-e, c-f : p= 0,005

Table 1: The apoptotic pecentages of the blasts in 0, 10 and 20 U/ml heparin concentrations at 0, 1 and 2 hours.

higher than those in 0 and 10 U/ml heparin concentrations (p=0.005). The highest apoptosis was determined in 20 U/ml heparin levels at first hour. A peak lower than 2n DNA (sub-G1 peak) that indicates the blasts ongoing to apoptosis was determined in 10 and 20 U/ml heparin concentrations at 0, 1 and 2 hours by FCM analyses. The

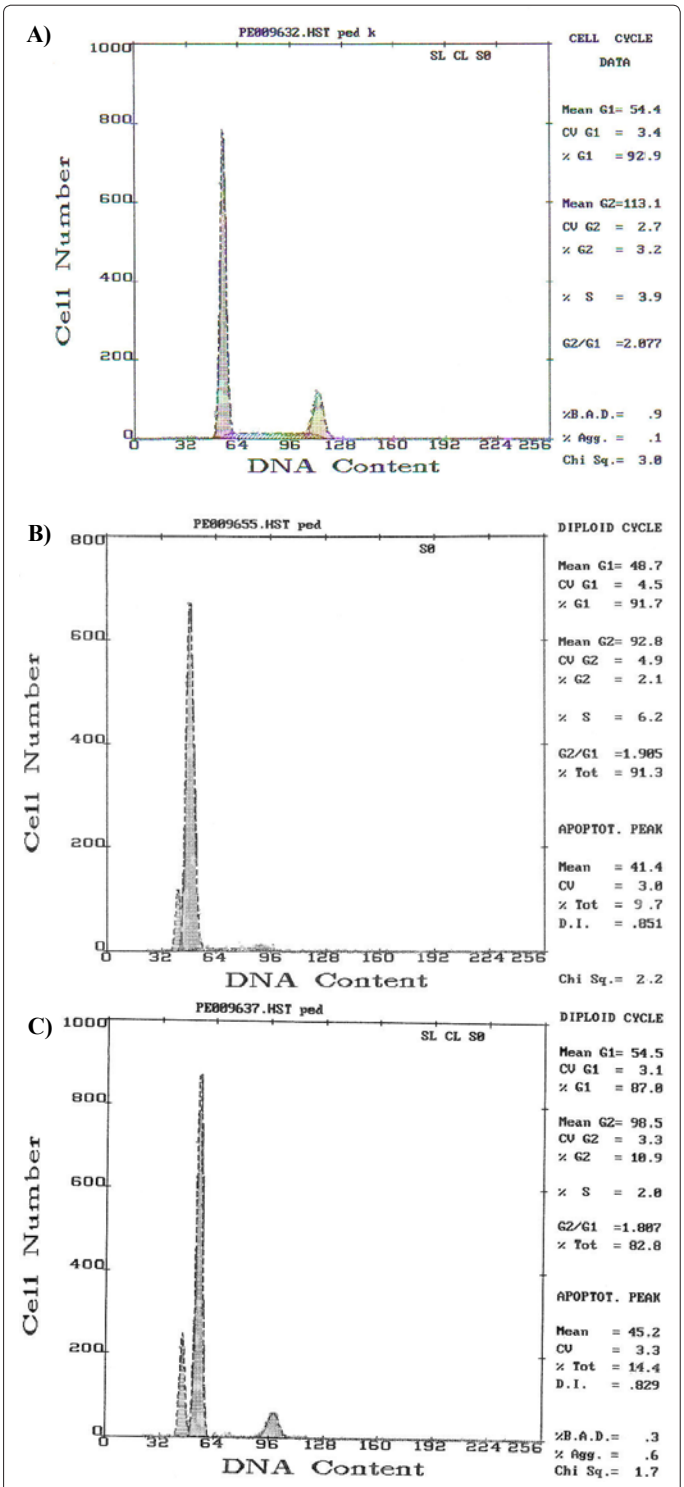

Figure 1: DNA histograms showing the percentage of apoptosis on the A) 0 U/ml, B) 10 U/ml, C) 20 U/ml heparin concentrations at first hour. Sub G_1 peak lower than DNA peak (left side of DNA peak) indicates the blast cells ongoing to apoptosis.

Heparin Time (hour)	0 U/ml (X ± SD) (min-max)	10 U/ml (X ± S.D) (min-max)	20 U/ml (X ± S.D) (min-max)
0	0.98 ± 0.35[a] 0.63 - 1.33	2.36 ± 0.34[d] 2.02 - 2.70	3.30 ± 0.32[g] 2.98 - 3.62
1	1.05 ± 0.28[b] 0,77 - 1.33	3.58 ± 0.43[e] 3.15 - 4.01	4.65 ± 0.34[h] 4.31 - 4.99
2	0.94 ± 0.24[c] 0.70 - 1.18	3.05 ± 0.24[f] 2.81 - 3.29	3.66 ± 0.23[i] 3.43 - 3.89

a-d, a-g, d-g, b-e, b-h, e-h, c-f, c-ı, f-ı, d-e, d-f, e-f, g-h, h-ı : p =0.005, a-c, a-b, b-c: p>0,05

Table 2: Intracellular Ca^{++} concentrations of the lymphoblasts treated with heparin in 0, 10 and 20 U/ml levels at 0, 1 and 2 hours after incubating with indo-1.

FCM histograms of a case whose lymphoblasts were incubated with heparin in 0, 10 and 20 U/ml concentrations at first hour are shown in Figure 1(A-C). The intracellular Ca^{++} levels of lymphoblasts incubated with indo-1 are shown in Table 2. Intracellular Ca^{++} levels at 1 and 2 hours were significantly higher than those at 0 hour in all heparin concentrations. Intracellular Ca^{++} levels in 20 U/ml heparin concentration at 0, 1 and 2 hours were significantly higher than those in 10 U/ml heparin concentrations (p=0.005). The highest intracellular calcium was determined 20 U/ml heparin levels at first hour.

Discussion

Kerr et al. [14] first described programmed cell death (apoptosis) and suggested that apoptosis was differently developed from the necrosis which is a pathologic and non-programmed event. Apoptosis develops under the genetic control following by the pathologic and/or physiologic stimulus [15,16]. Apoptosis has been determined by various methods. Although the electron microscopy is the most authentic method, it is an impracticable method to determinate apoptosis because apoptosis develops within a short-time and the cells are separately effected. The light microscopy is not an efficient method because it determines the apoptosis in 5 % of the cases [12]. The DNA gel agarose electrophoresis is an important method to show internucleosomal DNA fragmentation following by endogen endonuclease activation. The typical finding of apoptosis on the DNA gel electrophoresis is the ladder formation. The ladder formation was not determined while the cells were ongoing to apoptosis in a study and authors stated that the ladder formation could not to be a typical finding of apoptosis in every time [17]. The flow cytometry shows a sub-G_1 peak at the DNA analysis of the cells ongoing to apoptosis because the size and DNA content of the apoptotic cells decrease**.** The advantages of FCM are as follows: FCM is not influenced from cellular heterogeneity, a lot of cells are investigated at the same time and various investigations may be done in the selected cells. Therefore, we used FCM to detect the apoptosis in the study [4-6,12,13]. Annexin V binds Ca^{++} and phospholipids and it has been used in some investigations related to apoptosis. Phosphotidylserin expression on the cell which indicates the cell ongoing to apoptosis has been detected by Annexin V. Annexin V depends on the Ca^{++} concentration in the medium and the HEPES tampon included 1.8 M Ca^{++} or another tampon similar to the HEPES tampon should be used when Annexin V is used to detect the apoptosis. The little volumes of the diluted calcium in the medium make difficult the analysis of apoptosis by Annexin V. Propidium iodide differentiates the dead and the alive cells in the medium and determines the changes on the membrane of the cells ongoing to apoptosis in the early phase before just not to be the nucleus changes. Otherwise, PI

renders an opinion about the cell cycles and the chromatin changes. Therefore, we used PI instead of Annexin V to detect the apoptosis in our study [18]. The Fura-2, Indo-1, Fluo-3 and Rhod-3 dyes have been used to measure the intracellular Ca^{++} changes in the studies related to apoptosis. Indo-1 is an ideal dye to determine the intracellular Ca^{++} changes because Indo-1 is very sensitive to minor changes in the intracellular Ca^{++} concentrations. We therefore used Indo-1 dye to evaluate the intracellular Ca^{++} concentration in our study [19]. The intracellular Ca^{++} levels were assessed when the apoptotic effects of heparin in lymphoblasts were determined. The highest apoptotic peak was established in 20 U/ml heparin concentrations at first hour, as well [4-6]. The increases of the intracellular Ca^{++} levels were showed the parallelism with the increase of the percentages of apoptosis in 10 and 20 U/ml heparin concentrations at 1 and 2 hours ($p=0.005$). We first reported the apoptotic effect of heparin in lymphoblasts and found that the heparin induces apoptosis of the lymphoblasts in 5, 10 and 20 U/ml heparin levels at 1 and 2 hours, *in vitro*. Apoptosis was not determined 5, 10 and 20 U/ml heparin levels at 1 and 2 hours in the relapse ALL blasts. There were significant differences between the apoptosis percentages of lymphoblasts incubated with heparin of the new diagnosed patients and the relapse patients ($p<0.05$). The highest apoptosis was determined in 20 U/ml heparin levels at first hour. The apoptotic effects of heparin in neutrophils and mononuclear cells obtained from controls were established 20 U/ml heparin concentrations at 1 and 2 hours. The apoptotic effects of heparin in lymphoblasts were significantly higher than those in neutrophils and mononuclear cells in 5, 10 and 20 U/ml heparin levels at 1 and 2 hours ($p< 0.02$) [4]. In our another *in vitro* study, the apoptotic effects of heparin in 0, 10 and 20 U/ml concentrations and the expressions of fas and bcl-2 proteins in lymphoblasts were concomitantly determined by FCM analysis. Apoptosis was determined in 10 and 20 U/ml heparin levels at 1 and 2 hours and the highest apoptosis was detected in 20 U/ml heparin level at first hour. The fas expressions of the lymphoblasts incubated with heparin showed increase in 0, 10 and 20 U/ml heparin concentrations at 1 and 2 hours and the high fas expression levels were determined in 10 and 20 U/ml heparin concentrations at first hour ($p<0.01$). The bcl-2 expressions of the lymphoblasts were contrary decreased in the gradually increasing heparin concentrations and the low bcl-2 expressions were determined 10 and 20 U/ml heparin levels at first hour ($p<0.001$). We stated that the heparin induces apoptosis of lymphoblasts via the activation of the extrinsic pathway by the fas protein [5]. After that, we investigated whether or not the heparin induces apoptosis via activation of the caspase cascade involved in the intrinsic pathway of apoptosis, in-vitro and the statistically increase of the caspase-3 and -8 activities were observed in lymphoblasts incubated with heparin in 10 and 20 U/ml concentrations at 1 and 2 hours ($p<0.001$). We stated that heparin induces apoptosis of lymphoblasts via activation of the extrinsic pathway of apoptosis because of the increase of the caspase-8 activation [6]. Recently, caspase-9 activations and Cyt C levels were investigated in lymphoblasts incubated with heparin in 10 and 20 U/ml concentrations at 1 and 2 hours by FCM analysis. We claimed that heparin induces the apoptosis of lymphoblasts by the activation of the intrinsic pathway [8]. In the study, we proved the apoptotic effect of heparin in lymphoblasts by the activation of the intrinsic pathway of apoptosis. Once again and the high rates of apoptosis were determined in 10 and 20 U/ml heparin concentrations at first hour as seen in previous studies.

Castellot et al. [20] claimed that the vascular smooth muscle membrane expresses a special binding region of heparin and heparin prevents the proliferation of the smooth muscle cells. Heparin causes the increase of the c-myc, Bax, Bcl-2 protein expressions and induces apoptosis of the nasopharyngeal carcinoma cells [21]. Heparin attaches to the neutrophils in the irreversible, time-dependent and specific manners via a heparin-binding protein weighed 130 KD [22]. Leung et al. [23] stated that heparin adheres to the monocytes in the irreversible and specific manners. Unfortunately, we are not able to indicate a heparin-binding protein on the lymphoblasts.

This is the first report to investigate the relation between the increase of the intracellular Ca^{++} levels and the apoptotic effect of heparin in lymphoblasts. The increase of the cytosolic $Ca^{++}$and the decrease of the intracellular Ca^{++} storages were detected during the apoptotic process [9]. The bcl-2 is an antiapoptotic protein and induces apoptosis due to the increase of Cyt C release from the mitochondria. The protein bcl-2, associated with inositol 1,4,5 three phosphate (IP3) activates the calcium ion canals, increases the Ca^{++} concentration in the cytoplasm and induces apoptosis. Otherwise, the calcium ion canals are blocked when IP3 is inhibited and apoptosis is hindered [12]. The anti-apoptotic Bcl-X_L connects to the IP3 receptors and causes the blockage of the calcium ion canals and inhibition of apoptosis [24]. Splettstoesser et al. [25] suggested that Cisplatin causes the apoptotic effect on the HeLa-S3 cell lines to increase the intracellular Ca^{++} concentrations and 2-APB which is an IP3 blockage agent decreases the intracellular Ca^{++} levels. The endoplasmic reticulum and the mitochondria have an important role in apoptosis and this effect is provided by cellular Ca^{++} balance. The increase of the intracellular Ca^{++} levels causes the release of Cyt C from the mitochondria. Finally, caspase-9 and -3 activate the caspase-activated deoxyribonuclease turns into free form, the DNA fragmentation develops and the cell ongoing to apoptosis [26-29]. Consequently, we claimed that the heparin induces apoptosis in lymphoblasts following by the activation of the extrinsic pathway in our previous studies [5,6]. In the study, the statistically increasing intracellular Ca^{++} concentrations were found in 10 and 20 U/ml heparin levels at 1 and 2 hours compared to 0 U/ml heparin level at 0 hour ($p=0.005$). The apoptotic effect of heparin in lymphoblasts was indicated with the DNA analysis and concomitantly increased intracellular Ca^{++} concentrations. These findings indicate that heparin-induced apoptosis also develops due to the activation of the intrinsic pathway in addition to the activation of the extrinsic pathway.

The more comprehensive studies should be done to indicate whether or not heparin induces apoptosis in lymphoblasts following by the activation of the intrinsic pathway.

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