

Influence of blood sample storage and different types of anticoagulants on results of NK cytotoxicity tests

KATARZYNA POPKO¹, MONIKA PASKUDZKA², MAŁGORZATA PIEŚNIEWSKA³, SYLWIA DĄBROWSKA⁴, URSZULA DEMKOW¹, ANNA STELMASZCZYK-EMMEL¹

¹Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Poland

²Department of Laboratory Diagnostics, Medical University of Warsaw, Poland

³Student's Research Group at Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Poland

⁴Laboratory of Children's Hospital of Medical University of Warsaw, Poland

Abstract

Introduction: Natural killer (NK) cells are important players in the human immune response. Impaired NK function may lead to serious, life-threatening conditions. Defects may be consequences of genetic mutations or results of secondary factors such as infections, malignancies and autoimmune diseases. The cytotoxicity test is very useful, but its accessibility is limited to special immunological laboratories. Blood samples are often transported to remote centers, which takes time and requires special conditions.

The aim of this study was to compare cytotoxicity assay results between samples preserved with three different anticoagulants to standardize the diagnostic procedure.

Material and methods: Peripheral blood from healthy donors was taken with three anticoagulants: heparin, K₂EDTA and citrate. Peripheral blood mononuclear cells (PBMC) were isolated and tested directly after blood drawing and after 24-hour storage. Cytotoxic abilities of NK cells were tested in 4 h co-culture with K562. NK cytotoxicity was measured by flow cytometry.

Results: In most cases of analyzed healthy donors, cytotoxicity results were similar regardless of type of anticoagulant. However, the highest mean values were obtained in samples with citrate. There was a significant decrease in cytotoxicity after 24 hours of storage of the whole blood at ambient temperature. The mean drop in cytotoxicity results was substantial for all anticoagulants: 76% for heparin, 67% for citrate and 70% for EDTA.

Conclusions: Results of spontaneous NK cytotoxicity seem to be affected by the anticoagulants used for blood protection. Commercial instant cytotoxicity testing and delayed analysis after blood storage gave the highest results in blood with sodium citrate.

Key words: NK cells, cytotoxicity, EDTA, sodium citrate, heparin, flow cytometry.

(Cent Eur J Immunol 2023; 48 (4): 267-273)

Introduction

Natural killer cell function

Natural killer (NK) cells are a subset of mononuclear cells that play an important role in both innate and adaptive immune responses. NK cells are morphologically described as large granular lymphocytes (LGL) identified by the lack of CD3 (T-cell receptor) and surface expression of CD16 (Fcγ III receptor) and CD56 antigens [1]. By an assessment of CD56 and CD16 on the surface, NK cells are divided into two major subsets. The NK1 subset pres-

ents low expression of CD56 and high expression of CD16 (CD56^{dim}CD16⁺). NK1 cells comprise 90% of all NK cells in circulating blood and have stronger ability to kill target cells. Cells of the second subset, NK2, have higher expression of CD56 but they lack the CD16 receptor (CD56^{bright}CD16⁻). This population produces a greater amount of cytokines, such as interferon (IFN)-γ, compared with NK1 [2, 3].

The major biological function of NK cells is to recognize and kill tumor-transformed or virus-/bacteria-infected cells through the spontaneous cytotoxic effect. This effect

Correspondence: Katarzyna Popko, PhD, Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Poland, e-mail: katarzyna.popko@wum.edu.pl
Submitted: 20.12.2023, Accepted: 21.12.2023

is mainly mediated by cytotoxic granules containing sets of lytic enzymes (perforin, granzyme, TIA) and is regulated through the balance of stimulating and inhibiting signals, which are received by the NK cells through their receptors. Ligands for those receptors are mostly MHC class I molecules. When expression of MHC class I molecules is decreased, NK cells recognize the cell as a target and trigger cytotoxic mechanisms [4].

Cytotoxic abilities of NK cells can be used as one of the indicators of immune system efficiency. Decreased NK cytotoxicity may lead to immune dysfunctions and some diseases. Amongst the most serious systemic syndromes related to impaired NK functions hemophagocytic lymphohistiocytosis (HLH) is one of the most life-threatening conditions. HLH may be divided into two types: familial, which develops as a result of genetic mutation related strictly to NK function (FHL) or linked with other genetic diseases, e.g. Griscelli syndrome type 2 or Chediak-Higashi syndrome [12]; and acquired, resulting from viral infections, rheumatic diseases or other factors. If HLH is suspected the patient must be diagnosed in a special diagnostic center and his treatment needs to be monitored in specialist laboratories [13]. Sometimes samples are delivered from distant places, which is time consuming and may affect the results of activity tests.

The cytotoxicity test is a major and commonly used tool for testing NK activity. The long-time gold standard for assessing cell-mediated cytotoxic activity of NK cells is the chromium release assay (CRA). CRA involves radioactive labeling of target cells with ^{51}Cr and co-incubation with effector cells (K572, line of erythroleukemia cells). Although a number of non-radioactive assays have been created, most of them are based on the direct interaction between effector and target cells. For that reason, good quality of cells used in the test is crucial for credibility of the final results. The cytotoxic activity test can be carried out on isolated peripheral blood mononuclear cells (PBMC) or separated NK cells. Taking into consideration a good condition of the effector cells and previous suggestions that blood cells may act differently depending on the anticoagulant used for blood collection [7], the type of anticoagulant used for prevention of blood clotting should be carefully chosen. Anticoagulants commonly used for blood testing are sodium citrate, K_2EDTA and heparin.

Anticoagulants

K_2EDTA (ethylenediamine tetraacetic acid)

K_2EDTA is one of the most commonly used anticoagulants. It prevents blood from clotting through the binding of calcium ions. Calcium ion binding is convertible since metal complexes are created. Thus bound ions are able to exhibit diminished reactivity. Also other metals (II/second oxidation degree), e.g. copper, magnesium or iron, can be chelated in a 1 : 1 ratio by amino acid groups from K_2EDTA .

By this characteristic, K_2EDTA enables enzymes which have those metals in their active center. One calcium-dependent enzyme is phospholipase C. This enzyme contributes to many cells' activities, e.g. intracellular signaling, by producing 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, ion transportation and vesicle formation [8]. K_2EDTA also prevents oxidation of cholesterol and unsaturated fatty acids. Moreover, K_2EDTA provides protection of labile molecules such as cytokines and hormones [9].

Sodium citrate

The second well-known anticoagulant is sodium citrate. This anticoagulant has similar properties to K_2EDTA . Nevertheless, citrate chelates calcium ions by forming constant complexes. Not only calcium ions are bound by citrate, but also magnesium and zinc ions. Moreover, there are significant differences in total white blood count and total lymphocyte count between sodium citrate and EDTA. It has been reported that those parameters are decreased in blood samples drawn onto sodium citrate [10].

Heparin

Another anticoagulant used commonly in laboratory diagnostics is heparin. This mucopolysaccharide acts through binding with antithrombin and increasing its activity. Heparin is also able to bind with albumin, thrombin, von Willebrand factor and fibrinogen. Moreover, heparin stimulates release of some enzymes from platelets and granulocytes, e.g. elastase, lactoferrin, and myeloperoxidase. Heparin is able to inhibit the function of P- and L-selectin and has an affinity for platelet factor 4 and some other chemokines. Heparin can also inhibit the release of membrane-bound calcium mediated by the IP_3 pathway due to blocking the binding of IP_3 to its receptor. This results in higher intracellular calcium concentrations [11].

Aim of the study

The purpose of this study was to compare results of the cytotoxicity assay between samples taken from the same patient but with three different anticoagulants to standardize the diagnostic method. For this purpose we assessed and compared the effect of heparin, K_2EDTA and sodium citrate on the results of the cytotoxicity test, to evaluate which anticoagulant is the most appropriate for the tests. Moreover, we wanted to assess the influence of the time of blood storage on the results of the cytotoxicity assay.

Material and methods

Whole venous blood for this study was purchased from the Public Blood Bank in Warsaw.

Peripheral venous blood from twelve healthy donors 19 to 25 years old, both males and females, was drawn into

three tubes with different anticoagulants: heparin, K₂EDTA and citrate.

The flow cytometry cytotoxicity test was performed twice on the same blood sample at two time points. The first procedure was started one hour after blood drawing, and the measurement was done within 6 hours. The second procedure was started after 24 hours storage of the whole blood samples at room temperature, and the measurement was done within 6 hours.

Flow cytometry cytotoxicity test

Peripheral blood mononuclear cells were separated by density gradient centrifugation on Ficoll (Sigma-Aldrich), washed three times in 0.9% NaCl solution and suspended in RPMI medium supplemented with 10% FBS in a final concentration 4-6 million cells/ml.

The human erythroleukemia cell line K562 was used as an NK cell activator. Cells were cultured in RPMI medium (RPMI-1640 Medium, SIGMA-ALDRICH) with supplementation of 10% fetal bovine serum at 37°C under 5% CO₂. For the test K562 cells were stained with DiO (3,3'-dioctadecyloxycarbocyanine perchlorate) fluorescent dye for 30 minutes, washed twice with 0.9% NaCl solution and once with RPMI medium, then suspended in RPMI medium in a final concentration of 1.2 million cells/ml. The test was performed in a 96-well plate for each anticoagulant (Table 1).

The 96-well plate was centrifuged (1200 rpm for 5 minutes) and incubated for 4 hours at 37°C under 5% CO₂. After incubation cells were stained with propidium iodine (5 µl) for 30 minutes at 37°C under 5% CO₂ and analyzed in a flow cytometer.

Flow cytometry analysis

A two-parameter cytogram, log (FL1) vs. log (FL3), was plotted to discriminate the different cell types in the experiment. Acquisition was run to the final number of 10,000 events presenting DiO fluorescence.

Live target (K562) cells showed only DiO18 fluorescence, while dead target cells showed both DiO18 and PI fluorescence. Special gating was applied to obtain the number of DIO stained cells which also presented PI fluorescence. This population was considered to be killed by NK cells in a cytotoxic reaction (Fig. 1). The specific lysis index was presented as the percentage of dead target cells (cultured with effector cells) with subtracted dead target cells (cultured without effector cells).

Results

Results of cytotoxicity tests were presented as a percentage of target cells killed by effector cells throughout the 4 hours of incubation. Spontaneous mortality of target cells, assessed in the control tube, was subtracted from

the test results. The first cytotoxicity test was carried out within 6 hours from blood donation. The subsequent test was performed after 24-hour storage of the whole blood sample at ambient temperature. The mean values of cytotoxicity tests performed on the day of blood donation were comparable for all anticoagulants (Fig. 2). No statistically significant difference was noted.

Despite the fact that all the samples were taken from healthy volunteers, the standard deviations of cytotoxicity results were quite high. There was no significant difference in the value of cytotoxicity between all three samples. However, the highest mean values were obtained in samples with sodium citrate.

In all the cases the cytotoxicity decreased after 24-hour storage of the whole blood sample at ambient temperature (Fig. 3). The mean drop in cytotoxicity results was substantial for all anticoagulants: 76% for heparin, 67% for sodium citrate and 70% for EDTA.

There was a statistically significant decrease of the value of cytotoxicity in all samples after 24 h storage ($p < 0.01$) (Fig. 4).

The analysis of the scores of cytotoxicity in samples with different anticoagulants demonstrated that cells stored with citrate 3.2% presented the lowest cytotoxicity drop in comparison to heparin and K₂EDTA.

Conclusions

Time of the sample storage has a great impact on NK cells' cytotoxicity, leading to its significant decrease.

Sodium citrate is the most appropriate anticoagulant for tests performed 1 hour after blood taking and after 24 hours of blood storage.

Discussion

It has been reported that anticoagulants commonly used in laboratory medicine may have a significant impact on the results of some assays [14]. Although the cytotoxicity test is a major and commonly used tool for evaluation of NK cell function, there is no consensus across laboratories as to which anticoagulants should be used for blood protection. Standardization in blood preparation and storage is required to enable comparison of results obtained in different laboratories. Another factor which should be

Table 1. Scheme of reactions for blood samples with three different anticoagulants

Heparin	Sodium citrate	K ₂ EDTA
Control K562	Control K562	Control K562
Control PBMC	Control PBMC	Control PBMC
Test	Test	Test

K562 control contained only K562 cells and RPMI media. PBMC control contained PBMC and RPMI media. Test 1: K562 cells and PBMC (1 : 13).

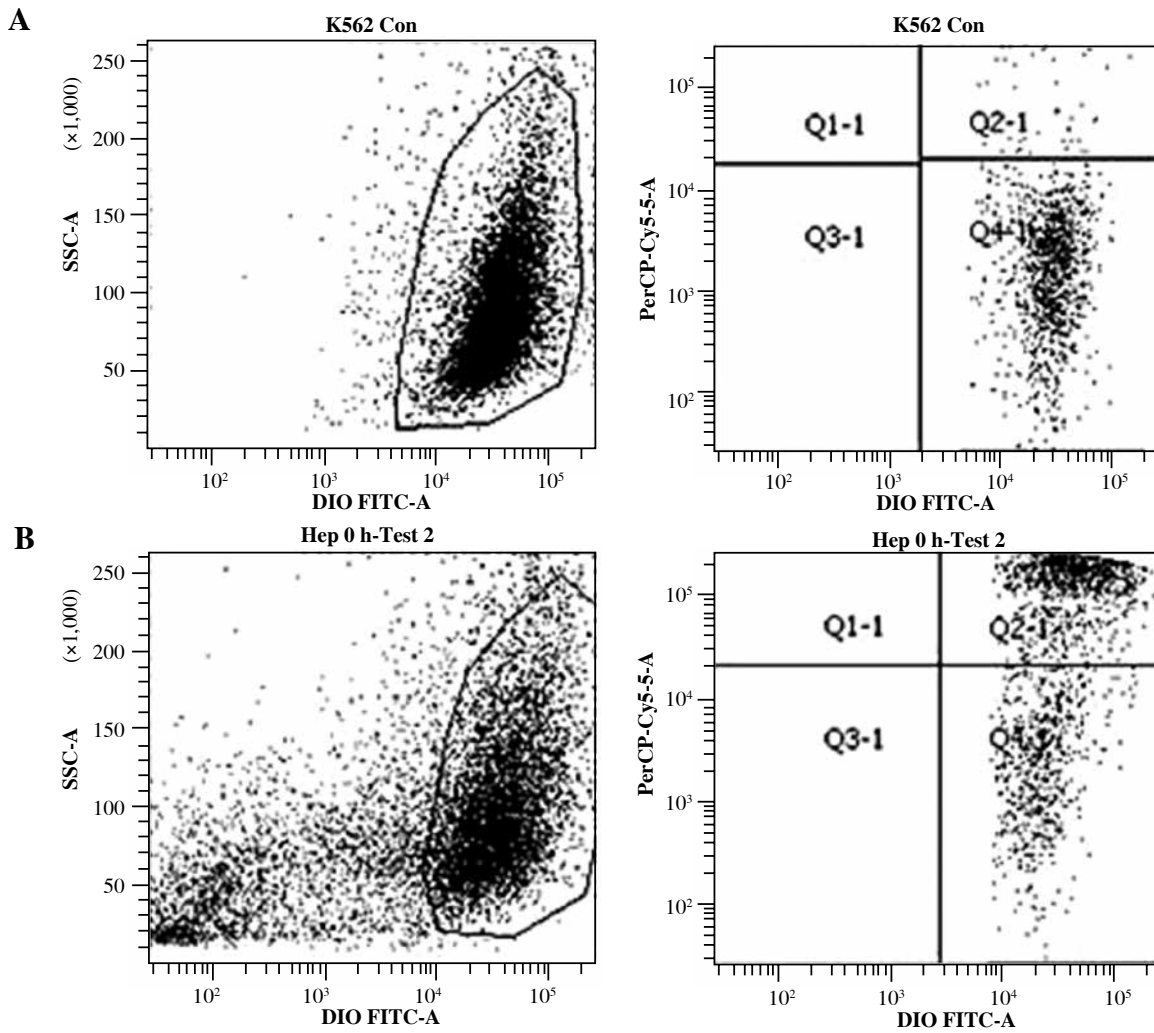
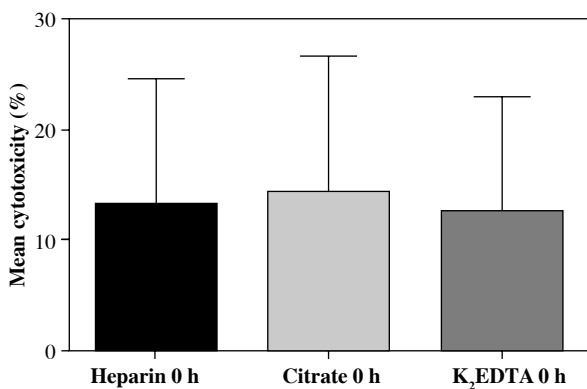
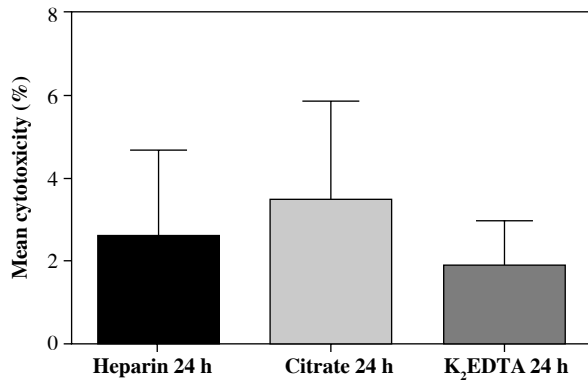


Fig. 1. Dotplots presenting gating applied in flow cytometry analysis of cytotoxicity test. A) Control sample, result after 4 h incubation of K562 cells, B) Test, result of 4 h co-incubation of K562 cells with PBMC



No.	Heparin	Citrate	K ₂ EDTA
1	3.4	4.6	4.0
2	3.6	1.5	1.3
3	17.8	20.5	20.6
4	17.2	26.8	22.0
5	3.5	3.8	11.2
6	12.6	12.2	12.2
7	8.0	6.8	4.4
8	7.6	11.8	3.6
9	25.5	39.4	31.3
10	42.4	31.0	28.1
11	10.1	10.4	9.2
12	5.4	3.1	1.6

Fig. 2. Values of cytotoxicity measurement in blood taken from 12 patients, with three different anticoagulants. Test performed within 6 hours after blood donation. Values of cytotoxicity in the table and the chart are presented as a percentage (%)



No.	Heparin	Citrate	K ₂ EDTA
1	2.0	1.1	0.3
2	0.6	1.1	1.2
3	3.6	6.8	2.7
4	5.7	4.8	3.8
5	0.4	0.6	0.7
6	3.0	5.0	2.4
7	1.7	6.1	2.2
8	2.6	4.2	2.1
9	7.3	5.9	2.9
10	2.0	2.0	2.5
11	1.1	3.8	0.6
12	1.2	0.2	1.1

Fig. 3. Values of cytotoxicity measurement in blood taken from 12 patients, with three different anticoagulants. Test performed after 24 hours from blood donation. Values of cytotoxicity in the table and the chart are presented as a percentage (%)

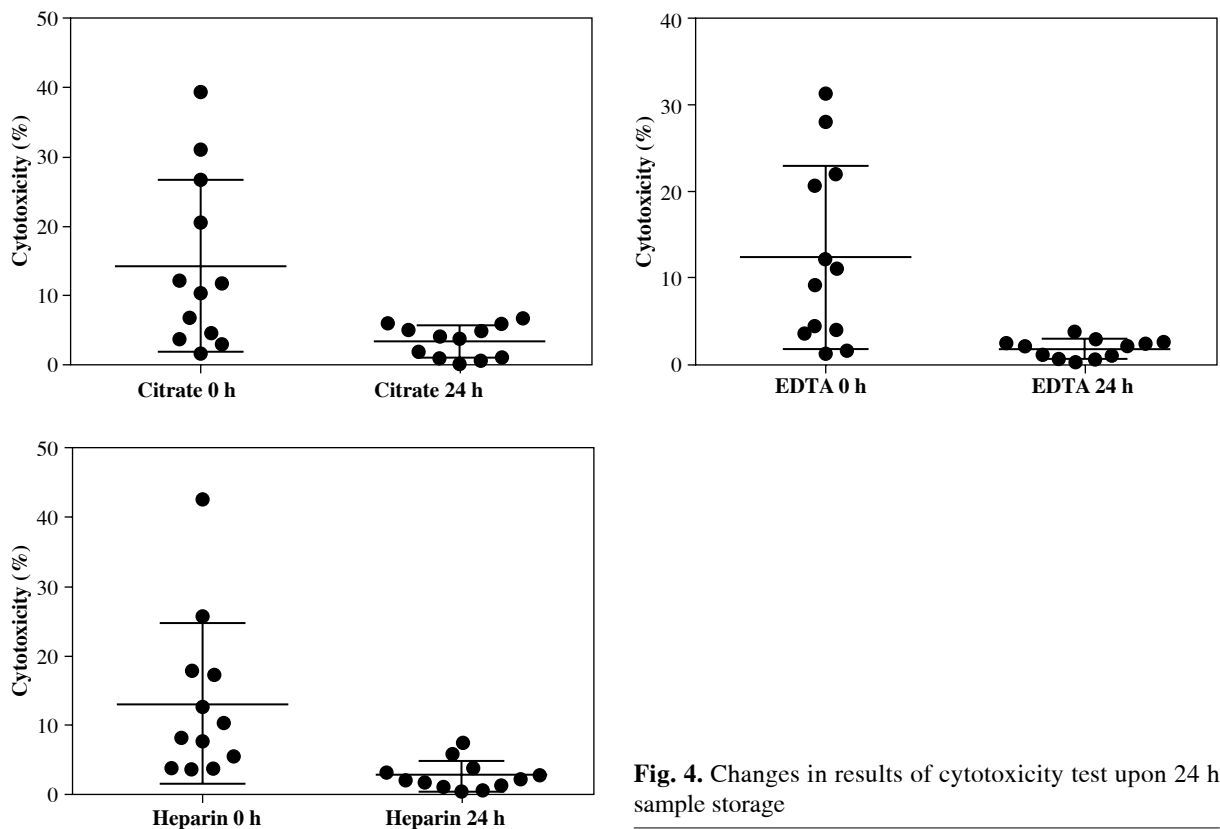


Fig. 4. Changes in results of cytotoxicity test upon 24 h sample storage

taken into consideration regarding cytotoxicity test results is the time of sample delivery to the laboratory. Since the method of NK cytotoxicity measurement is quite complicated and requires specific laboratory equipment, it is available only in specialized immunological laboratories.

In this study we compared cytotoxicity results measured in blood samples drawn with three most popular anticoagulants at the time of blood donation and after 24 hours of storage at ambient temperature. According to our

findings, sodium citrate seems to have the smallest impact on the results of the cytotoxicity test.

There may be a few reasons for the differences in the cytotoxicity results regarding anticoagulants added to the blood sample. The capacity of NK cells for spontaneous cytotoxicity against target cells may be modified by the anticoagulant used. There is some scientific evidence indicating that different anticoagulants may affect the process of PBMC isolation [7]. In one of the studies blood samples

with heparin and citrate were analyzed with an ultrasonic scanner. Heparinized blood revealed considerable cell and platelet aggregation. This phenomenon was less visible in blood sample with citrate [20]. We did not notice such an influence in our study, but an impact of these substances on the number of NK cells after isolation in the total population of PBMC cannot be excluded. A correlation between the percentage of NK cells in circulation and results of the cytotoxicity test was previously found [19]. The separation technique used in our study (with Ficoll Sigma-Aldrich) can remove not only granulocytes to acquire PBMC but also dead lymphoid cells from the specimen (including NK cells), which may have contributed to decreased cytotoxicity after 24 hours [15].

It is a common knowledge that Ca^{2+} is a key metabolic factor for NK function including spontaneous cytotoxic reaction against target cells. It was recently reported that cytotoxicity of NK cells is strongly dependent on Ca^{2+} influx from the extracellular medium [21]. Later findings led to identification of Orai1 channels which are required for exocytosis and target cell killing by primary human NK [22]. Taking into consideration that reactivity of cells and intracellular signaling depends largely on the concentration of calcium ions, the use of EDTA and citrate, which bind free calcium from the environment, may affect cell activity. Studies on neutrophils showed that the intracellular concentration of calcium ions was considerably lower in samples with EDTA than in heparin or citrate. Activation of neutrophils measured by the assessment of oxidative burst was also lower in samples with EDTA than those with citrate and heparin [7]. It is in line with our observations, where cytotoxicity was the highest in samples with citrate and the lowest in those with EDTA.

Anticoagulants may also affect NK reactivity through modification of interleukin secretion. It was reported that interleukin (IL)-1 concentrations in PBMC separated from blood with EDTA were lower than those with citrate or heparin [16]. Although we observed higher cytotoxicity in samples with citrate and heparin than with EDTA, it has been found in previous studies that granzyme b levels were higher in blood samples anticoagulated with EDTA than with heparin [17]. Although we did not measure IL-1 concentration in our study, its impact as a proinflammatory cytokine on NK cell activity cannot be excluded.

The influence of blood sample storage and type of anticoagulant (heparin and EDTA) on NK cell activity in whole blood was also analyzed by Son and collaborators. They observed that NK cytotoxicity was higher in heparinized blood, which is similar to our results. They also evaluated NK activity in freshly drawn blood and blood stored for 18 hours. In both cases heparin was a better anticoagulant for this test [18].

According to our findings, and in line with other studies on this subject, sodium citrate seems to be the best anticoagulant for assessment of NK cytotoxicity. However it

should be underlined that long blood sample storage before testing has a significant influence on the results, observed as decreased cytotoxicity.

The authors declare no conflict of interest.

References

1. Aktas E, Kucuksezer UC, Bilgic S, et al. (2009): Relationship between CD107a expression and cytotoxic activity. *Cell Immunol* 254: 149-154.
2. Boissel L, Tuncer HH, Betancur M, et al. (2008): Umbilical cord mesenchymal stem cells increase expansion of cord blood natural killer cells. *Biol Blood Marrow Transplant* 14: 1031-1038.
3. Cichocki F, Sitnicka E, Bryceson YT (2014): NK cell development and function – Plasticity and redundancy unleashed. *Semin Immunol* 26: 114-126.
4. Bonavida B (2014): NK cell phenotypic and functional heterogeneities and molecular mechanisms of cytotoxicity. *Crit Rev Oncog* 19: 21-45.
5. Jang YY, Cho D, Kim SK, et al. (2012): An improved flow cytometry-based natural killer cytotoxicity assay involving calcein AM staining of effector cells. *Ann Clin Lab Sci* 42: 42-49.
6. McBride JE, Meaney MP, John C, et al. (2017): Flow cytometric analysis of natural killer cell lytic activity in human whole blood. *J Vis Exp* 121: 54779.
7. Freitas M, Porto G, Lima JL, Fernandes E (2008): Isolation and activation of human neutrophils in vitro. The importance of the anticoagulant used during blood collection. *Clin Biochem* 41: 570-575.
8. Szumilo M, Rahden-Staroń I (2008): Phosphoinositide-specific phospholipase C in mammalian cells: Structure, properties, and function. *Postepy Hig Med Dosw* 62: 47-54.
9. Banfi G, Salvagno GL, Lippi G (2007): The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes. *Clin Chem Lab Med* 45: 565-576.
10. Sharif MT, Mahabadi MA, Moshfeghi S, et al. (2012): Artfactual changes in hematological variables in equine blood samples stored at different temperatures and various anticoagulants. *Comp Clin Path* 21: 449-452.
11. Engstad CS, Gutteberg TJ, Osterud B (1997): Modulation of blood cell activation by four commonly used anticoagulants. *Thromb Haemost* 77: 690-696.
12. Shabrish S, Gupta M, Madkaikar M (2016): A modified NK cell degranulation assay applicable for routine evaluation of NK cell function. *J Immunol Res* 2016: 3769590.
13. Martínez I, Fernández L, Valentín J, et al. Natural killer cell cytotoxic activity in critical pediatric patients with suspected hemophagocytic syndrome. *Med Intensiva* 39: 213-221.
14. Morrison BJ, Martin NJ, Rehman T, et al. (2018): Influence of sample collection tube method, anticoagulant-containing plasma versus serum, on influenza virus hemagglutination inhibition titer and microneutralization titer serological assays. *BMC Health Serv Res* 18: 651.
15. Dagur PK, McCoy JP (2016): Collection, storage, and preparation of human blood cells HHS public access. *Curr Protoc Cytom* 73: 5-6.

16. Aziz N, Butch AW, Ryner TC, et al. (2018): The influence of EDTA Vacutainer blood collection tube on the level of blood interleukin-1 receptor antagonist. *J Immunol Methods* 464: 114-118.
17. Scholman RC, Giovannone B, Hiddingh S, et al. (2018): Effect of anticoagulants on 162 circulating immune related proteins in healthy subjects. *Cytokine* 106: 114-124.
18. Son BK, Roberts LR, Ank BJ, Stiehm ER (1996): Effects of anticoagulant, serum, and temperature on the natural killer activity of human peripheral blood mononuclear cells stored overnight. *Clin Diagn Lab Immunol* 3: 260-264.
19. Popko K, Malinowska I, Rychta M, et al. (2013): Frequency and spontaneous cytotoxicity of natural killer cells in healthy children: preliminary results. *Cent Eur J Immunol* 38: 562-568.
20. Mahony C, Ferguson J (1992): The effect of heparin versus citrate on blood echogenicity in vitro: the role of platelet and platelet-neutrophil aggregates. *Ultrasound Med Biol* 18: 851-859.
21. Golstein P, Smith ET (1976): The lethal hit stage of mouse T and non-T cell-mediated cytotoxicity: differences in cation requirements and characterization of an analytical "cation pulse" method. *Eur J Immunol* 6: 31-37.
22. Maul-Pavicic A, Chiang SC, Rensing-Ehl A, et al. (2011): ORA11-mediated calcium influx is required for human cytotoxic lymphocyte degranulation and target cell lysis. *Proc Natl Acad Sci USA* 108: 3324-3329.