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ORIGINAL RESEARCH

Analysis of Oxidative Burst Capacity of Neutrophils in Buffy-Coat Concentrates*

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ABSTRACT

Buffy-Coat concentrates (BFCs) can be transfused to provide neutrophil support in patients with febrile neutropenia. In this study, the oxidative burst capacity of neutrophils in BFC was investigated in 12 hours-stored BFCs (n=16). Samples taken from healthy volunteers (n=7) (fresh EDTA; fEDTA) and taken before donation from blood donors who donated BFCs (n=16) (donor EDTA; dEDTA) were included. fEDTA and dEDTA samples were used to compare the effect of time. Nitroblue tetrazolium (NBT) test was performed to evaluate the oxidative burst. Different titrations (1:16, 1:32, 1:64, 1:128) of phorbol myristate acetate (PMA) were used. BFC samples without PMA addition were used as negative control (NC). While similar results were obtained in BFC samples with fEDTA samples in NC, oxidative activity was statistically high in the dEDTA samples. These results suggested that neutrophils in dEDTA may be spontaneously activated. In all PMA titrations except 1:64 PMA titers, the oxidative burst capacity in dEDTA and BFC samples was significantly lower than that in fEDTA samples. Also, oxidative burst significantly decreased in fEDTA-dEDTA-BFC in all PMA titrations. Spontaneous burst was detected in dEDTA samples under PMA-free conditions, but not in BFC samples, indicating that the additive solution in BFC protected neutrophils from spontaneous activation. Despite the statistically significant decrease, neutrophils in BFCs can remain functional due to their high oxidative activity at all titers (above 75%). This indicates that they can function when transfused. This result also shows that we can use BFCs as a source of neutrophils in experiments.

Keywords: Buffy-Coat Concentrates. Nitroblue Tetrazolium Test. Oxidative Burst. Neutrophil.

Buffy-Coat Konsantrelerindeki Nötrofillerin Oksidatif Patlama Kapasitelerinin Analizi

ÖZET

Buffy-Coat konsantreleri (BFC'ler) febril nötropenisi olan hastalarda nötrofil desteği sağlamak için transfüze edilmektedir. Bu çalışmada, BFC'deki nötrofillerin oksidatif patlama kapasitesi araştırıldı. 12 saat depolanmış BFC'ler (n=16), sağlıklı gönüllülerden alınan örnekler (n=7) (taze EDTA; fEDTA) ve BFC bağışlayan kan bağışçılarından bağış öncesi alınan örnekler (n=16) (donör EDTA; dEDTA) çalışmaya dahil edildi. fEDTA ve dEDTA örnekleri zamanın oksidatif patlama üzerindeki etkisini değerlendirmek amacıyla çalışmaya dahil edildi. Oksidatif patlamayı değerlendirmek için Nitroblue tetrazolium (NBT) testi farklı titrasyonlarda phorbol miristat asetat (PMA) ile gerçekleştirildi. PMA'nın 1:1, 1:2, 1:4, 1:8 titrasyonlarında oksidatif patlama %95'ten yüksek bulunduğundan, çalışmaya 1:16, 1:32, 1:64, 1:128 titrasyonları ile devam edildi. Ayrıca PMA eklenmemiş BFC örnekleri negatif kontrol olarak kullanıldı. Negatif kontroldeki fEDTA örnekleri ile BFC örneklerinde benzer oksidatif patlama düzeyleri belirlenirken, dEDTA örneklerinde oksidatif aktivite istatistiksel olarak yüksek bulundu. Bu sonuçlar dEDTA'da bulunan nötrofillerin spontan olarak aktive olabileceğini düşündürdü. dEDTA ve BFC örneklerindeki oksidatif patlama kapasitesi 1:64 PMA titrasyonu hariç tüm PMA titrasyonlarında fEDTA örneklerine göre anlamlı düzeyde düşük bulundu. Ayrıca artan titrasyona bağlı olarak oksidatif kapasitenin tüm gruplarda önemli ölçüde azaldığı tespit edildi. PMA'sız dEDTA örneklerinde spontan patlama tespit edilirken BFC örneklerinde tespit edilmemesi, BFC'deki ek solüsyonun nötrofilleri spontan aktivasyondan koruduğunu göstermektedir. fEDTA örneklerine göre anlamlı azalmaya rağmen, BFC'lerdeki nötrofillerin tüm titrelerde (%75'in üzerinde) yüksek oksidatif aktivite göstermeleri işlevsel kalabildiklerini düşünülmektedir. Elde edilen sonuçlar BFC içindeki nötrofillerin transfüzyon yapıldığında işlev görebileceklerini düşündürmekte, ayrıca deneylerde BFC'leri nötrofil kaynağı olarak kullanabileceğimizi göstermektedir.

Anahtar Kelimeler: Buffy-Coat Konsantreleri. Nitroblue Tetrazolium Testi. Oksidatif Patlama. Nötrofil.

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Neutrophils, also called polymorphonuclear cells (PMNCs), differentiate from granulocyte-monocyte progenitor cells in the bone marrow and are the first cells to arrive at the site of infection[1]. They form the first defence against bacterial and fungal infections[2]. They migrate toward the site of inflammation under the guidance of chemokines and cytokines. They fight pathogens by digesting microbes in phagolysosomes through oxidative burst and reactive oxygen species (ROS) production at the site of inflammation, releasing antimicrobial peptides in their granules and/or using Neutrophil Extracellular Traps that immobilise and kill microorganisms and activate other immune cells[2,3]. One of these pathways, phagocytosis, is a special form of receptor-mediated endocytosis used by neutrophils and macrophages to engulf microbes[4]. In phagocytosis, a pathogen is taken into the cell by neutrophils and transferred to the phagosome[5]. Neutrophils digest microorganisms in phagosomes through the ROS they produce and microbicidal proteins in their granules, such as myeloperoxidase, neutrophil elastase, and matrix metalloproteinases[6]. Therefore, analysing ROS production levels is one of the accepted methods to evaluate the function of neutrophils.

Febrile neutropenia (FN) and neutrophil dysfunction (ND), characterized by a decrease in the absolute number of PMNCs in peripheral blood below 500/mm^3, pose a problem in immunocompromised individuals, especially in the fight against bacterial and fungal infections. Despite the low-grade evidence about the effects of prophylactic granulocyte transfusions[7,8], granulocyte concentrate (GC) transfusions have been used in patients with FN and ND for many years[9,10]. Although apheresis GC (aGC) is beneficial, its obtaining process is expensive and time-consuming. For this reason, buffy coat-derived granulocyte concentrates (BFC) could be an alternative[11–13]. BFCs are a by-product rich in lymphocytes, monocytes, granulocytes, and platelets. They are usually thrown away in situations other than FN and ND. However, if they are to be transfused, they must be used within 24 hours of collection due to the decrease in pH and cell survival[14].

The aim of this study was to investigate the neutrophil oxidative burst level in BFCs. BFCs with a median shelf life of 12 hours were selected for the analysis. The changes in the oxidative burst capacity of these BFCs according to different concentrations of stimuli were analyzed. In this way, it was investigated whether the oxidative burst capacity of neutrophils in BFCs stored for 12 hours would be sufficient for the transfused patient. The BFC results were compared with the oxidative burst capacities of fresh neutrophils to obtain information about product sufficiency. In addition, another aim of the study was to evaluate whether BFCs could be used as a neutrophil source for neutrophil-related experiments.

Material and Method

Preparation of Buffy-Coat Concentrates (BFCs)

Blood components (erythrocyte concentrate, platelet concentrate, and plasma) were separated from whole blood with the Reveos Automated Blood Processing System (Terumo, Lakewood, CO, USA). The BFCs occurring in this process were included in our study (n= 16)

Donor blood samples (Donor EDTA; dEDTA)

EDTA blood samples were taken from the blood donor before blood donation and stored for 12 hours. The samples (BFCs and dEDTA) in our study belongs the same donor and same donation (n=16).

Fresh blood samples (Fresh EDTA; fEDTA)

EDTA blood samples obtained from volunteers immediately before the Nitroblue Tetrazolium Test (NBT) were used. The effects of the 12 hours of storage were evaluated by comparing these fresh samples (n=7) with dEDTAs and BFCs.

Analyses of Leukocyte Counts

Leukocyte counts in all BFC, dEDTA and fEDTA samples were tested with a hematology analyzer (CellDyn 1800, Abbott Diagnostic, Chicago, Illunois, USA).

Nitroblue Tetrazolium Tests

NBT test is the gold standard method to measure oxidative burst and was used also in our study. Tests were performed for 100.000 neutrophils. Phorbol 12-myristate 13-acetate (PMA) (Merck; Sigma-Aldrich;

Darmstadt, Germany; Cat no: P8139) was used to stimulate neutrophils. NBT solutions with and without PMA were used in the analyses. To prepare NBT solution "without PMA; PMA-free", 15 mL NaCl, 18.75 mg NBT, and 1.275 µL human albumin were mixed. After 10 mL of this mixture was separated as PMA-free NBT solution, 25 µL PMA was added to the remaining 5 mL. This NBT solution with PMA was then titrated with the NBT solution PMA-free to obtain NBT solutions containing eight different doses of PMA (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128). Since the oxidative burst in PMA titrations at 1:1, 1:2, 1:4, and 1:8 was higher than 95%, the study was continued with titrations at 1:16, 1:32, 1:64, and 1:128. The first six experiments were carried out with 1:16, 1:32, and 1:64 PMA titrations; the next three experiments with 1:32, 1:6,4 and 1:128; and the last seven experiments with all titrations. An equal volume of NBT solution was added to the blood samples for analysis and kept in the incubator for 20 minutes and at room temperature for 10 minutes. The samples were spread on slides as a thin layer and fixed with ethyl alcohol after drying. After the alcohol was dried, the slides were stained with Giemsa stain and kept for 20 minutes, then washed with distilled water and left to dry. After the slides were dried, they were evaluated under the light microscope at 100x magnification.

Statistical Analysis

Descriptive statistics are reported as percentage. Mann-Whitney U and Wilcoxon signed rank tests were used to compare the groups. The data analysis was performed using GraphPad Prism. The significance level was established as $p<0.05$.

Results

Leukocyte Counts

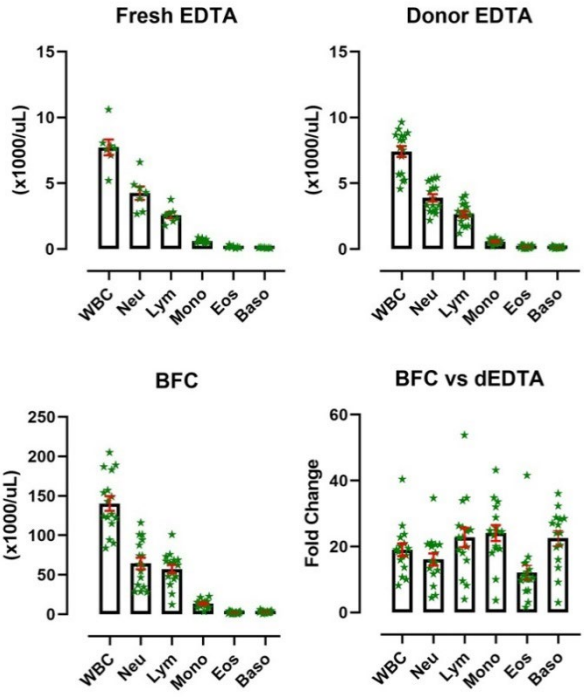
WBCs were 7.68±0.75 K/µL in fEDTA samples and 7.42±1.63 K/µL in dEDTA samples. Approximately 50% of these cells were neutrophils, and 35% were lymphocytes. In BFCs, 140.39±37.1 K/µL WBCs were measured, and approximately 45% were neutrophils and 42% were lymphocytes. It was calculated that the number of WBCs in BFCs increased approximately 19-fold, the number of neutrophils 16-fold, and the number of lymphocytes 23-fold compared to the donor's own dEDTA samples (Figure 1).

Nitroblue Tetrazolium Tests

The oxidative burst capacities of neutrophils in the absence of PMA and 1:16, 1:32, 1:64, and 1:128 titrations of PMA were evaluated. Interestingly, it was determined that dEDTAs led a significantly higher oxidative burst than BFC samples under PMA-free conditions ($p<0.05$) (Figure 2). Oxidative burst was found to decrease in fEDTA, dEDTA, and BFC samples in advanced titrations compared to 1:16 PMA. In all three groups, it was significantly decreased at 1:64 PMA titre compared to 1:16. In addition, statistically significant decreases were detected in PMA titres of 1:32 in fEDTA samples and 1:128 in BFC samples *($p<0.05$)* (Figure 2). Except for the 1:64 dose, oxidative bursts were statistically significantly decreased in dEDTA and BFC samples compared to fEDTA in all other titrations *($p<0.05$)* (Figure 3).

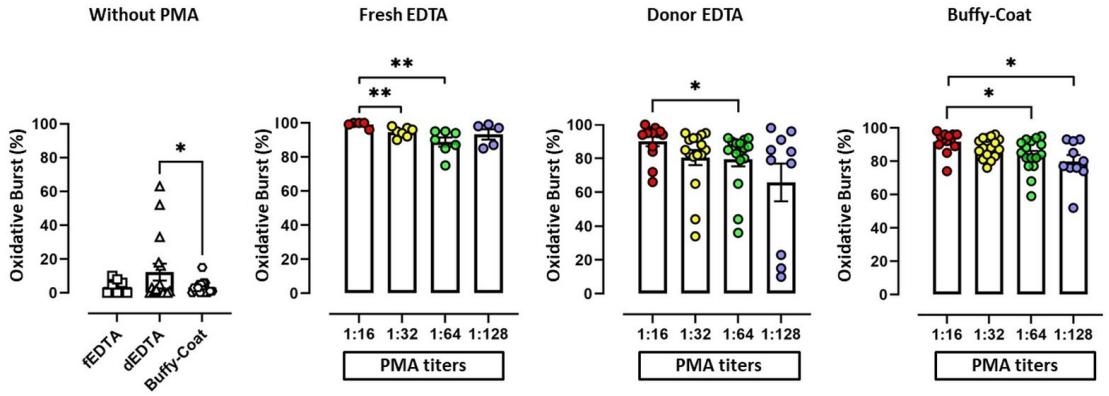
No significant difference was found when BFC samples were compared with dEDTA samples (Figure 4A). While no oxidative burst less than 50% was detected in BFC samples, it was detected in three dEDTA samples (Figure 4B). The levels of one of them were low only at 1:128, while the levels of the other two were low at 1:32, 1:64 and 1:128 titrations.

When the last seven experiments having all PMA titration results were analyzed, there was no significant difference between dETAs and BFSs (Figure 5). Only one dEDTA sample at 1:128 showed a very low oxidative burst.



WBC: White Blood Cell; BFC: Buffy-Coat concentrate; fEDTA: fresh EDTA; dEDTA: donor EDTA

Figure 1:
WBC counts in fEDTA, dEDTA and BFC.



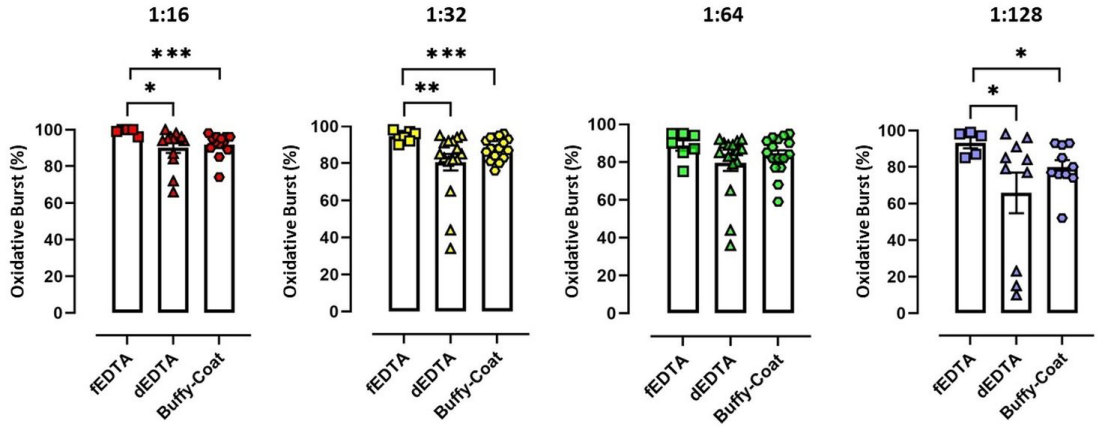
fEDTA samples were tested five times each at 1:16 and 1:128 PMA titres and seven times each at 1:32 and 1:64; dEDTA and BFC samples were tested thirteen times at 1:16, sixteen times each at 1:32 and 1:64, and ten times at 1:128.

BFC: Buffy-Coat concentrate; fEDTA: fresh EDTA; dEDTA: donor EDTA

** 0.05>p≥0.01, ** 0.01>p≥0.005*

Figure 2:

Oxidative bursts without PMA and titrated PMAs in fEDTA, dEDTA and BFC.



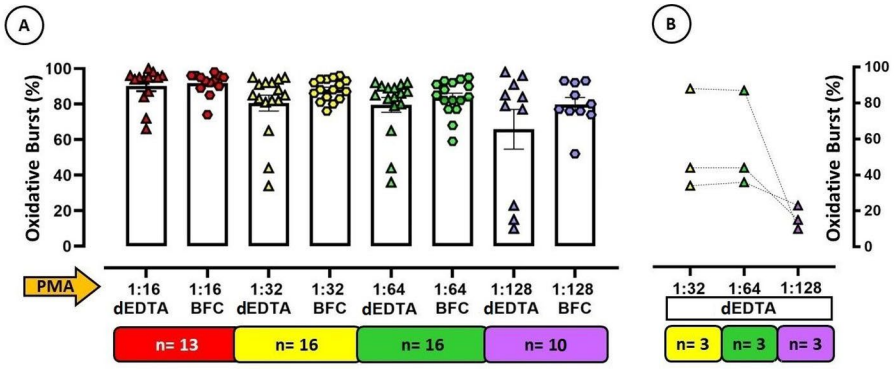
fEDTA samples were tested five times each at 1:16 and 1:128 PMA titres and seven times each at 1:32 and 1:64; dEDTA and BFC samples were tested thirteen times at 1:16, sixteen times each at 1:32 and 1:64, and ten times at 1:128.

BFC: Buffy-Coat concentrate; fEDTA: fresh EDTA; dEDTA: donor EDTA

** 0.05>p≥0.01, ** 0.01>p≥0.005, *** 0.005>p≥0.001*

Figure 3:

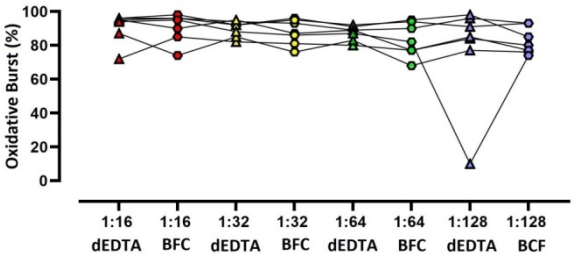
Oxidative bursts in each titer of PMA. Comparing the fEDTA, dEDTA, and BFC.



BFC: Buffy-Coat concentrate; fEDTA: fresh EDTA; dEDTA: donor EDTA

Figure 4:

dEDTA and BFC results A) Comparing the dEDTA and BFC results in all PMA titers B) Three samples of dEDTAs showing low level oxidative burst (< 50%).



BFC: Buffy-Coat concentrate; fEDTA: fresh EDTA; dEDTA: donor EDTA

Figure 5:
The results of seven dEDTA and BFC samples having results in all PMA titers

Discussion and Conclusion

BFCs are considered an alternative to aGCs in acute situations due to some disadvantages of aGCs such as donors' preconditioning process, time-consuming apheresis process, and requirement of the use of some chemicals such as hydroxyethyl starch[11–14]. BFC transfusion offers advantages in terms of time, cost and donor reactions. Also, it was noticed that BFC transfusion was safe but not associated with survival benefits[11]. Many studies driven to detect BFC safety have investigated some properties of neutrophils, such as viability, phagocytosis, oxidative burst, and chemotaxis[14,15]. These studies have shown that BFCs offer a valuable alternative to aGCs. In our study, oxidative burst decreased in relation to both storage (fEDTA versus dEDTA and BFC) time and PMA titer. Although oxidative burst was found to be lower in BFC than in fEDTA, it was seen to be above 75% even at a PMA titer of 1:128. This result indicates that the oxidative burst in BFC is markedly maintained at 12 hours storage, and BFCs can be transfused with functional expectation, as shown in previous studies.

All GCs should be transfused within 24 hours after collection owing to the rapid decrease in pH and cell survival. Increasing lactate levels is one of the main reasons for decreasing cell survival[14]. However, the existance of red blood cells and platelets into the BFC can reduce granulocyte viability, oxidative burst and phagocytic activity[14,15]. The decrease in oxidative burst found in our study may be related to the red blood cells and platelets present in BFCs. Since BFCs are used in our hospital without any purification process, this study was performed with BFCs containing red blood cells and platelets.

Efforts are being made to extend the storage time of GCs. Pooling of BFCs, purification of granulocytes, and use of additive solutions are some of the topics covered in this effort. Storage time can be extended to 72 hours through these topics without decreasing the viability, phagocytosis, or oxidative burst[14–16]. Additive solutions have been shown to participate in this effect[15]. In our study, the fact that oxidative burst was found significantly higher in dEDTA samples than BFC in the "without PMA" group may be an indicator of the additive solutions effect (CPD; Citrate-Phospate-Dextrose) within the BFCs. CPD might have protected the granulocytes from a spontaneous burst in BFC.

This study showed that granulocytes in BFCs could be stimulated to an acceptable level (above 75 percent) even with low-titre PMA. In addition, the additive solution (CPD) appears to preserve the oxidative burst capacity of granulocyte in BFC. Thus, it is thought that granulocytes in BFC may provide oxidative burst support to FN and ND patients. Also, it has been reported that approximately $1x10^{10}$ granulocytes should be transfused to FN and ND patients[14]. To achieve this target, 15-20 BFCs must transfused. Our results show that our BFCs contain sufficient amount of granulocytes. This result also shows that we can use BFCs that have been stored for at least 12 hours as a neutrophil source in experiments. Additionally, the 1:64 PMA titre appears to be a better option for NBT tests. The reason why the oxidative burst capacity of the dEDTA and BFC samples was significantly lower than that of the fEDTA samples, except for the 1:64 titres, is not understood. However, it was thought that this result was not related to the experimental design. As the values were close for all samples in this titration, the 1:64 PMA titre may be the titre of choice in future studies evaluating the effects of different molecules on neutrophil oxidative burst.

The strengths of our study include systematically examining the oxidative burst capacity at different PMA concentrations and comparing it with fresh samples. However, the study focused on a single storage period (12 hours), and the effects of different storage periods were not analyzed. This constitutes its limitation. Future studies should examine the effects of different storage periods and conditions on neutrophil function more comprehensively.

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Idea and design: S.H.B.;
Data collection and processing: S.H.B., H.A., İ.Ö.;
Analysis and interpretation of data: S.H.B., D.Y.E., Y.H., H.B.O.;
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