DETERMINATION OF THE NUMBER AND VIABILITY OF WHITE BLOOD CELLS IN CAPILLARY AND VENOSE BLOOD

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Abstract

Determination of the number of white blood cells (WBCs) and their viability in capillary or venous blood is an important indicator for clinical diagnosis of patients. A method has been developed to determine the cell number and viability of white blood cells in fresh capillary or venous blood by a new EASYCOUNTER BC fluorescence microscope (produced by Milkotronic LTD). Concentration and viability of WBCs in capillary and venous blood can be measured without the removal of red blood cells from the sample, as opposed to the often used microscopic counting in which the erythrocyte lysis is obligatory condition. A new DNA dye Sofia Green is used. The dye permeates only in dead cells because their cell membrane is compromised. Living cells do not stain because their cell membrane is intact. Therefore, in order to determine the total number of leukocytes, it is necessary to add white blood cell lysis solution to make them permeable to the Sofia Green dye. In this case all white blood cells are stained by the Sofia Green dye. The optimal working range for measuring WBCs with the EASYCOUNTER BC was determined. The coefficient of variation (CV, %) of the results obtained with EASYCOUNTER BC in the optimal working range varied between 3-4%. For comparison the CV of the results obtained by microscopic counting was significantly higher, in the range 12-26%. This shows that the EASYCOUNTER BC fluorescence microscope possess better reproducibility and accuracy and could be used for rapid clinical diagnosis.

***Key words**: white blood cells, DNA dye, cell counting, automatic fluorescence microscope*

INTRODUCTION

Determination of the number of white blood cells (WBCs) as well as their viability in capillary or venous blood is an important indicator for the treatment of patients. This analysis is necessary to diagnose infections, allergic reactions, inflammation, blood cancers such as leukemia or lymphoma, side effects caused by drugs and monitoring of treatment. The results of the analysis are desirable to be obtained as soon as possible in order for the doctor to decide quickly for diagnosis and treatment. Determining the number of white blood cells is one of the most common tests that are carried out with patients when their diagnosis is established. Leukocytes are found throughout the body, including the blood and lymph system, and are far less than red blood cells [5]. They protect the body against infectious diseases, other foreign agents, altered cells of the body [2, 6-8]. As a reference or quantitative limits in adults and adolescents (over 15 years of age), values between 3.5 and 10.3 x 10^9/ l are considered normal. Increased leucocyte level is present in various infections, mainly bacterial. Their values can reach 30.0 x 10^9/ l. All white blood cells have a nucleus that distinguishes them from red blood cells (RBCs) and platelets and can therefore be colored when mixed with DNA dyes.

Generally, the number of white blood cells is obtained by manual procedure, by coloring the blood sample with Giemsa stain (methylviolet, gentian violet) and microscopic examination of the sample in a hemocytometer (Buker's Chamber).The method has high standard error due to differences in the ability of a person to assess the intensity of colors and counting errors.

The implementation of automated cell counting devices to quantify the viable number of cells is a much more convenient method due to the removal of human factor [1,3,4]. Replacing the Giemsa dye with a fluorescent dye increase the sensitivity of the analysis. The fluorescent dye is bound to dead cell DNA and thus the number of dead cells is determined, and by the difference of the total number of cells

and the listed dead cells the number of living cells is determined and the cell viability is estimated. Therefore, there is a great need for a quick and simple method for white blood cells counting. In addition, devices that can be seamlessly mounted in a variety of clinical applications and even in remote field clinics are needed to provide a quick and accurate analysis.

MATERIALS AND METHODS

Materials

Capillary or venous blood sample was taken from volunteers; for the preparation of lysis buffer for WBCs: Citric acid monohydrate, Octylphenol ethoxylate (Triton X-100), and Trisodium citrate dehydrate were used and purchased from Sigma Aldrich. For the preparation of lysis buffer for RBCs : acetic acid, gentian violet from Sigma Aldrich were used. DNA fluorescent dye Sofia Green, manufactured by Milkotronic LTD, was also used.

Apparatus

Automatic fluorescent microscope Easycounter BC equipped with special software, microfluid camera (cellchip) for sample loading and analysing, (Milkotronic, LTD, Bulgaria), Olympus BX51 Microscope.

Methods

Determination of total cell count and viability of WBCs in capillary and venous blood using EASYCOUNTER BC

The fresh capillary or venous blood was diluted with PBS in a ratio of 1: 4 (Dilution factor 5). 100 μl of the diluted blood were pipetted and place in an eppendorf tube containing lyophilized mixture of leukocyte lysis reagent (1,7 % sodium citrate dehydrate, 0,81 % citric acid, 1 % Triton %) and Sofia Green fluorescent dye (10 μg/ml). The sample was incubated for 10 minutes at room temperature. The prepared sample was used for determination of WBCs total count.

Separately, 100 μl of undiluted capillary or venous blood were pipetted in another eppendorf tube containing only the lyophilized DNA fluorescent dye Sofia Green (2 μg/ml). This sample was used for determination of dead WBCs count.

After that 8 μl from each samples were placed in channel A (total cell count) and B (dead cell count) of the microfluidic camera (cellchip).

Counting and analyzing the total number of WBCs, the number of dead WBCs and the WBCs viability was measured and calculated using the EASYCOUNTER BC.

Determination of the total count of WBCs using light microscope and lysis of the red blood cells

RBCs affect the correct count of WBCs due to their high count in blood. For this purpose, to 20 μl of capillary or venous blood sample 180 μl of Turk's reagent were added, the mixture was placed in a hemocytometer (Bürker counting chamber), and WBCs were counted under low magnification [9]. The number of white blood cells was obtained by bright field microscopic examination of the sample.

RESULTS AND DISCUSSION

Determination of the viability of WBCs in capillary and venous blood using EASYCOUNTER BC

Measurement of the concentration and viability of nucleated cells in clinical samples is difficult to achieve due to the presence of red blood cells (RBCs) and RBCs cellular debris. To remove interfering red blood cells, clinical samples are usually pretreated in order to lyse red blood cells or Ficoll gradient dividing is performed [10]. But even these methods are not always successful in removing all the red blood cells and lead to significant errors in determining white blood cells concentration.

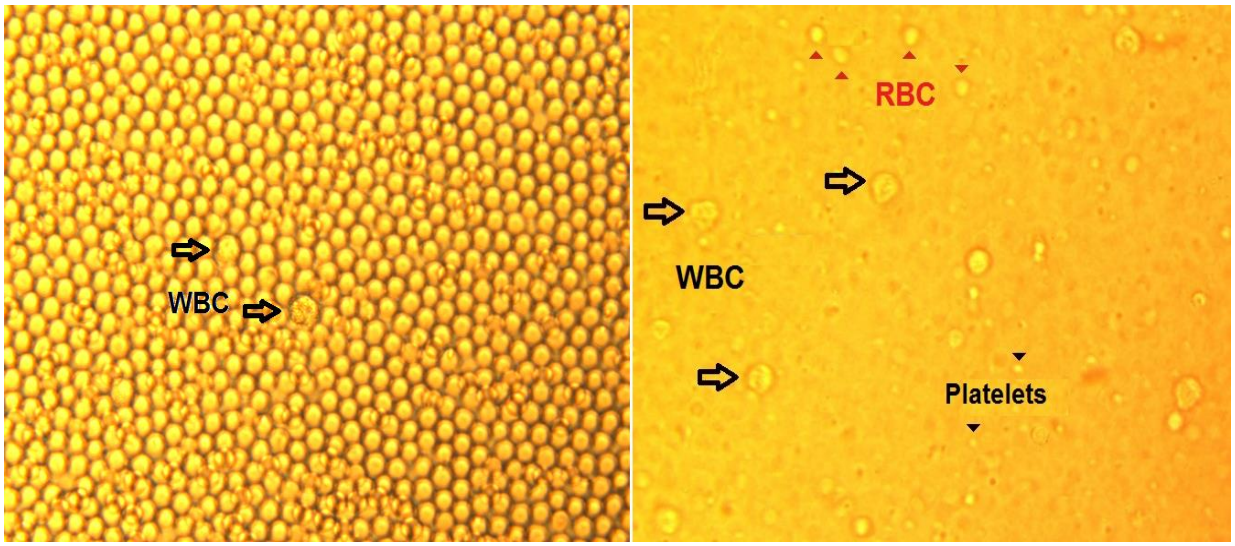

Figure 1. Bright field images from light microscope of: (left) diluted blood sample (without RBCs lysis), (right) diluted blood sample with lysed RBCs (x 40).

On figure 1 images of blood sample under microscope are shown. On the left the blood sample is diluted 10 times with PBS, and is not treated in order to remove the RBCs. Their count in the sample is so high that greatly hinders the WBCs observation and make their correct and accurate counting impossible. Obviously at high red cells count, the number of observed and counted WBCs is decreased. That is why the RBCs lysis before WBCs microscopic counting is a must.

On the right the blood sample is diluted 10 times with 2 % aqueous solution of acetic acid in order to remove the RBCs. The image shows that there is a great decrease in the number of RBCs, which facilitates the WBCs counting. Nevertheless there are residual RBCs that could still interfere the correct WBCs counting.

Using the cell fluorescence method, the need for red blood cells lysis and Ficoll gradient dividing is eliminated. Thus, the concentration and viability of WBCs in capillary and venous blood can be measured without the removal of red blood cells from the sample. For determination of the total and dead white blood cells count in a blood sample different dilution factor is used. Measurement is performed directly with the EASYCOUNTER BC automatic fluorescence microscope. Based on these two results and the built-in software, the viability of the leukocytes is calculated as a percentage. The DNA dye Sofia Green stains only the nuclei of dead leukocytes because their cell membrane is compromised and permeable to the dye (Figure 2 (right)). Live cells do not stain because their cell membrane is impermeable to the dye. To determine the total leukocyte count, it is necessary to add white cell lysis solution to the cells to make them permeable to the Sofia Green DNA dye and to stain the nuclei of all cells (Figure 2 (left)).

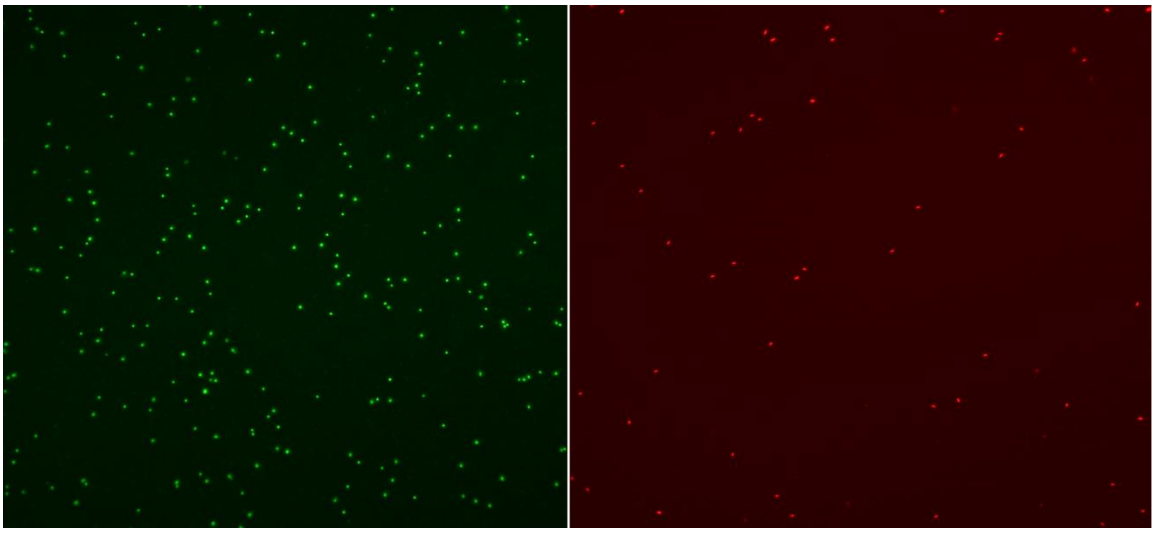

Figure 2. Images of stained WBCs - total count (left) and stained dead WBCs (right) obtained by the automatic fluorescence microscope Easycounter BC

Usually, the number of white blood dead cells in a fresh sample is very small, and if the sample is diluted, a large measurement error will occur. The possibility of separately measuring these two parameters allows the appropriate dilution of the samples for dead and total number measurement so that they fall within the optimum measurement range of the device and thus the obtained results are with high accuracy and precision.

Validation of automatic fluorescence microscopic method for total WBCs count using EASYCOUNTER BC

The total number of WBCs in 10 samples of venous blood, diluted in PBS in a ratio of 1: 4, was determined. White blood cell lysis reagent and Sofia Green fluorescent dye DNA were used. After the respective incubation, counting and analyzing the total number of WBCs using EASYCOUNTER BC was performed. In order to validate the method, the same samples of venous blood were treated in order to lyse the RBCs. Then a visual microscopic examination was performed using Bürker counting chamber.

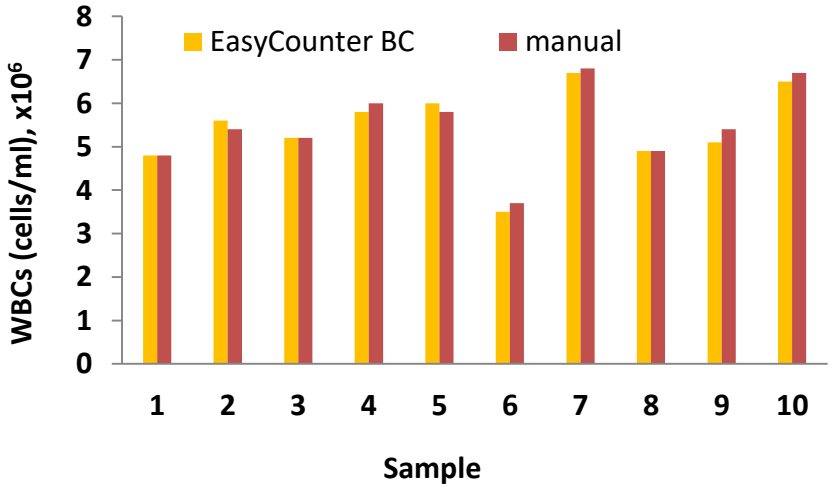

Figure 3. Total number of WSCs determined by EASYCOUNTER BC and manual light microscopic counting

Figure 3 shows that the results obtained by the two methods are comparable. But the developed automatic fluorescence method has a number of advantages - it does not require red blood cells lysis, takes less time and has lower standard error. Due to the bigger sample volume that is loaded in the cellchip thousands of leukocytes are counted by the automated techniques in order to obtain more accurate results, whereas in visual examination using hemocytometer 100-200 white blood cells are typically tested.

The optimal concentration range for measuring WBCs with EASYCOUNTER BC was determined. For this purpose, a series of diluted capillary blood was prepared using PBS. Each diluted sample was treated with lysis reagent for white blood cells and was stained with the DNA fluorescent dye Sofia Green. After counting and analyzing the total number of WBCs in all the diluted samples, using the EASYCOUNTER BC (each sample was measured 10 times) the graphical dependency between the dilution factor and the total number of cells per ml was plotted. The linear range of the curve defining the optimal cell count interval was determined. The results are presented on Figure 4.

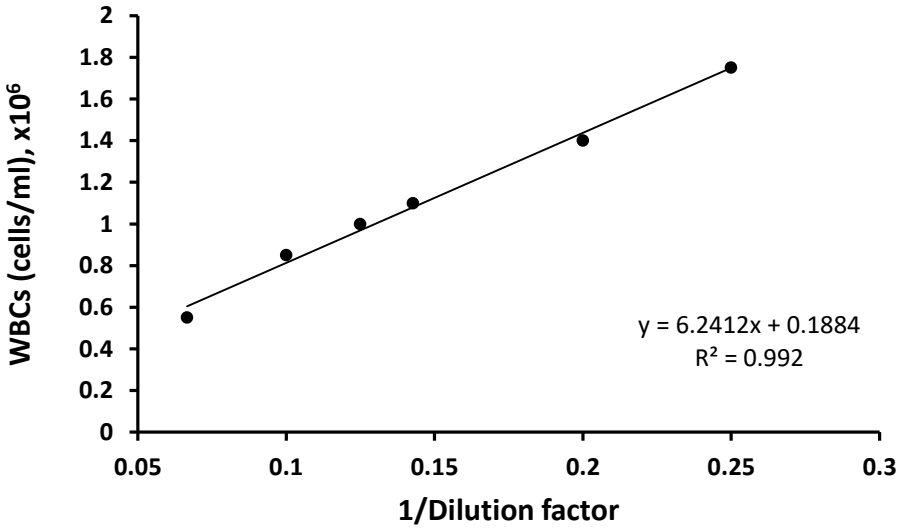

Figure 4. Linear range of measurement of blood sample at different dilutions

From Fig. 4 it is obvious that the optimal linear range for WBC counting is from $4x10^5$ to $2.5x10^6$ cells per ml. The coefficients of variation of the results obtained using Easycounter BC were determined. The WBCs count of the same sample was determined using manual light microscopic counting and the coefficients of variance were calculated. Comparison of the coefficients of variation obtained by the two methods shows that the CVs obtained by the automatic fluorescence microscopic method are considerably lower than those obtained by the optical microscopic counting method (Table 1). When counting the cells with Easycounter BC in the optimum concentration range CV moves within the 3-4% range. In the microscopic hand counting method CV is significantly higher 11-26%. This shows the better reproducibility and accuracy of the automatic fluorescence microscopic method.

Table 1. Comparison of the coefficient of variation (CV,%) of WBCs counting by Easycounter BC and by Manual microscopic method

WBCs (cells/ml)	Dilution factor	CV, %	
		Easycounter BC	Manual
3,5 $X10^6$	2	7.40	-
1,75 $X10^6$	4	3.02	11
1,40 $X10^6$	5	3.44	12
1,00 $X10^6$	7	3.97	13
0,87$X10^6$	8	4.07	15
0,70 $X10^6$	10	5.20	18
0,46 $X10^6$	15	5.80	21
0,35 $X10^6$	20	7.50	26
0,23 $X10^6$	30	8.30	-

CONCLUSION

The proposed method for WBCs counting using the fluorescent DNA dye Sofia Green and the automatic fluorescent microscope provides fast, accurate and precision results. Using the cell fluorescence method, the need for red blood cells lysis is eliminated. Thus, the concentration and viability of WBCs in capillary and venous blood can be measured without the removal of red blood cells from the sample. The ready to use lyophilized reagents and the specific software makes the assay very easy to perform. The assay allows to eliminate the human error and presents the results in a very accessible form. The method could be successfully incorporated in different aspect of clinical diagnostics.

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