Influence of five different methods for blood neutrophil separation

Zlatina Becheva, Tzonka Godjevargova

Abstract: *Five different methods for blood cell sedimentation for neutrophil obtaining were studied. Dextran and PVP in different concentrations were used. The Ficoll density gradient was applied. After that the microscopic analysis and neutrophil elastase – substrate reaction were used to determine the neutrophil amount in each layer. It was defined that the most suitable method for rapid obtaining neutrophils was the PVP blood sedimentation.*

Key words: *Dextran, PVP, Ficoll, blood cells, PMN, neutrophil elastase.*

INTRODUCTION

Blood cell separation techniques are widely used in medicine and science laboratories. As Arne Bøyum explains, cells have different properties: density (d) and size (or radius = r), which determine the sedimentation rate (u) in a liquid medium. Impact of these factors is shown in general sedimentation law (equation 1) [4].

(1)

G – centrifugal acceleration or acceleration of gravity;

d_0 – density of the medium;

η – viscosity of the medium through which the particles (cells) falls.

Intrinsic viscosity is calculated with the Mark-Houwink equation (equation 2) [1, 2].

(2)

K and a – constants for a given polymer type;

M – molecular weight.

Different sedimentation agents react in different way with blood cells. Armstrong et al. explain that aggregation of the blood cells is possible with polyoxyethylene (POE) molecule weight $\geq$ 15 000 Da, polyvinylpyrrolidone (PVP) $\geq$ 20 000 Da, Dextran $\geq$ 40 000 Da. Otherwise the sedimentation agent would inhibit the cell aggregation [1].

PVP (M = 29 000 Da) and Dextran (M = 500 000 Da) for obtaining blood cells – neutrophils was used in this paper. These cells are the soldiers forming the first line of defense against foreign invaders [7]. They are polymorphonuclear neutrophils, which contained active endopeptidase – elastase in their azurophilic granules. This enzyme is typical for neutrophils [10].

In this study five different ways for blood sedimentation was investigated – 1:5 = 6% Dextran : blood; 1:1 = 6% Dextran : blood; 1:1 = 6% PVP : blood; 1:1 = 3% PVP : blood; 1:1= 1% PVP : blood. The advantages and disadvantages of each method for neutrophil obtaining were discussed.

MATERIALS AND METHODS

Reagents and Chemicals

Sodium citrate, Dextran 500, PVP, NaCl, NH_4Cl, Ficoll-Paque PREMIUM, methylene blue, N-succinyl-Ala-Ala-Ala-p-nitroanilide, Triton X 100, Tris-HCl were delivered from Sigma-Aldrich (Germany). Water was purified with ELGA PURELAB Option.

Bovine blood

Bovine blood (2 300 mL) was obtained from three healthy heifers. They had no mastitis or other inflammatory symptoms. The blood was mixed with anticoagulant sodium citrate (3.8% solution, 9:1 ratio).

Blood sedimentation methods

Sedimentation was made with Dextran 500 and PVP (29 kDa) as sedimentation agents [1, 4]. They were diluted to different concentrations with normal saline solution.

Five sedimentation models were done:

• 1:5 = 6% Dextran : blood (model A)

• 1:1 = 6% Dextran : blood (model B)
• 1:1 = 6% PVP : blood (model C)
• 1:1 = 3% PVP : blood (model D)
• 1:1 = 1% PVP : blood (model E)

The same size sterile beakers with inner diameter 8.5 cm were used. Total volume in beakers was equal (600 mL). The solutions were let settled for 24 hours at room temperature in sterile conditions. There were three layers (1- upper, 2 - middle and 3 - bottom) in each model after incubation time for blood sedimentation.

White blood pellet obtaining

All layers were gently collected in different glasses, so 15 samples were obtained. NH_4Cl with concentration 0.87% (3:1 ratio) was used for erythrocyte lysis in each sample. They were centrifuged three times in plastic tubes at 600 x g, 15 min, 4°C to obtain white pellets. Then pellets were washed with PBS (pH 7.4, 10 mM) [4, 9]. After that they were defined by weight and stored at -4°C. The pellets were examined for elastase activity by specific substrate reaction.

Elastase – substrate reaction with white blood pellets

Colorimetric method for determination of neutrophil elastase activity with N-succinyl-Ala-Ala-Ala-p-nitroanilide as a specific substrate was used. First, suspensions of 0.1g white blood pellets in 1 mL normal saline solution were made. Permeable buffer was made with 0.2% Triton X 100 in Tris-HCl buffer (0.1M; pH 8) [5]. Substrate solution was made with concentration 0,0044M in Tris-HCl buffer (0.1M; pH 8) [3, 6]. After that, 50 µL pellet-saline suspensions in 50 µL permeable buffer were incubated in microplate for 15 min at room temperature. Then 200 µL substrate solution was added to the mixture. Absorption was measured at 405 nm wavelength in microplate reader Rayto RT-2100C.

Blood cell separation by density gradient

The neutrophils were isolated from pellets by Ficoll density gradient. Pellet-saline suspensions were made by mixing 0.1g white blood pellet (from the layer with elastase activity) with 1 mL normal saline solution. The gradient was made according to the manufacturer's instructions. Briefly, 3 mL Ficoll-Paque PREMIUM was added to centrifuge tube (10 mL capacity). Carefully 4 mL of the pellet-saline suspension on Ficoll-Paque PREMIUM were layered (figure 1A) and spin at 400 x g for 30 min at 20°C. Four different layers were obtained (figure 1B) [9, 11].

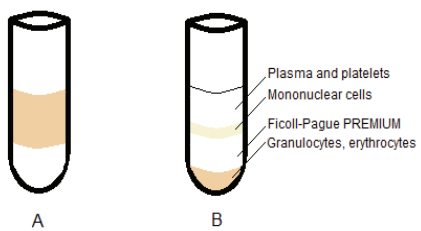

Fig.1. (A) Ficoll-pellets before centrifugation, (B) Ficoll-pellets after centrifugation.

To demonstrate the presence of neutrophils in these layers we made microscopic analysis.

Microscopic analysis of the layers

Olympus BX51 microscope, equipped with QImaging Retiga 2000R camera, was used for cell observation. Mononuclear cells and granulocytes, obtained with Ficoll density gradient, were colored on standard technology with methylene blue [8]. Also pictures were taken of the pellets, obtained in *White blood pellet obtaining* step, with no dye.

RESULTS AND DISCUSSION

Blood sedimentation methods.

Five models (A, B, C, D and E) for blood sedimentation were made. The time required for precipitation was long because the blood volume was large. Inner diameter of the beakers was equal and the total loaded blood too. This provided equal conditions for sedimentation of the different models. All models (A-E) had three layers after the incubation time. Upper layer was pale yellow, middle layer was light red and bottom layer was deep red. The percents of these layers were different. For example, after incubation time pale yellow layer was: model A – 12%; model B – 28.87%; model C – 21.51%; model D – 22.51%; model E – 28.14%. The layers in model B and model E were observed after 6 hours, but for other models after 24h. Blood without sedimentation agent and no incubation time (model F) was used for comparison. Figure 2 is a scheme of the models.

White blood pellet obtaining

Erythrocyte lysis of the sixteen samples was made (5 x 3 samples with sedimentation agent and 1 only blood). The obtained white pellets by centrifugation were examined by weight. There were great differences in total amount of the pellets in each model (table 1).

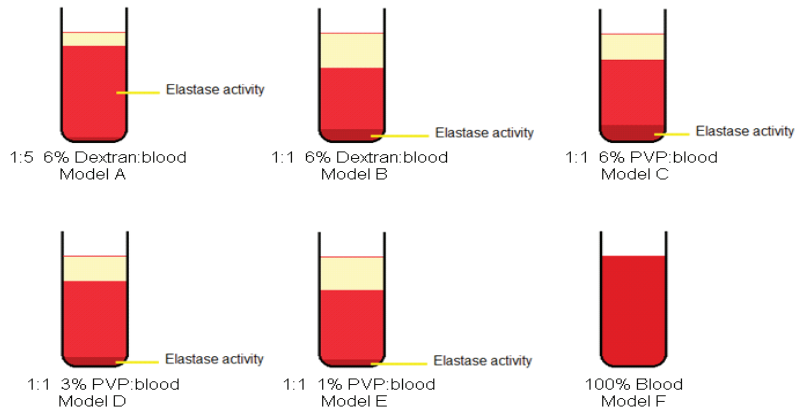

Fig. 2. Five different models for blood sedimentation (A, B, C, D and E) and comparison model (F).

Table 1. White pellets founded in three layers in models (A, B, C, D, E) with sedimentation agents and in model F without sedimentation agent

Model	Layer	Pellets, g
A	Upper	0.0765
	Middle	3.4138
	Bottom	0.4571
B	Upper	0.1979
	Middle	2.3628
	Bottom	1.7885
C	Upper	0.0863
	Middle	1.2580
	Bottom	3.6490
D	Upper	0.0775
	Middle	0.8532
	Bottom	4.6151
E	Upper	0.0970
	Middle	1.7866
	Bottom	1.3130
F	-	1.3620

Differences were due to use of different concentrations of sedimentation agents and various ratio agent:blood. .Also there was a large difference in obtained pellets in the separate layers in the same model. It was sign that all blood cells (including neutrophils) were grouped in a different single layer. For example, in model D the bottom layer was 4.6151 g, and upper and middle layer were less than 1 g. The presence of different blood cells in the pellets was demonstrated by Ficoll-Paque PREMIUM density gradient.

Elastase – substrate reaction with white blood pellets

All pellet samples were examined for elastase activity to proof the neutrophil presence. Neutrophils were in the bottom layer in model B, C, D and E, and in the middle layer of the model A (figure 2). Further processing was made with the layers with elastase activity. Elastrase activity values were in the range 0.254 - 0.946 AU. The highest activity had bottom layer of model E (1:1 1%PVP : blood). Absorption of this layer was 0.946 AU at 405nm wavelength. Therefore the most suitable agent was 1%PVP and the optimal ratio was 1:1 (1%PVP : blood).

Blood cell separation by density gradient

Pellets with elastase activity were divided by Ficoll, which created density gradient. In the separation liquid mononuclear cells (monocytes and lymphocytes) are in the buffy coat, because they have a lower density. The granulocytes and erythrocytes which have a higher density fall to the bottom [4]. The highest content of clear neutrophils, from the pellets with elastase activity, was in bottom layers from models E (1:1 1%PVP : blood) and B (1:1 6%Dextran : blood).

Microscopic analysis of the layers

Methylene blue is a blue dye, which colored nucleuses in the cells and the whole cell becomes visible at an appropriate magnification (in this study magnification is 400x). Figure 3 is microscopic photos of the obtained layers after Ficoll density gradient separation of the blood cells in bottom layer of model D dyed with methylene blue. Figure 3A is mononuclear cell layer. Figure 3B is granulocytes and erythrocytes layer. The last layer contained only granulocytes because in the step *White blood pellet obtaining* was made erythrocyte lysis.

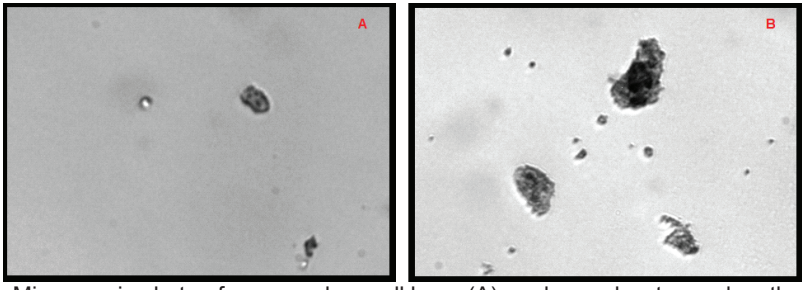

Fig. 3. Microscopic photo of mononuclear cell layer (A), and granulocytes and erythrocytes layer (B) after Ficoll density gradient separation, dyed with methylene blue.

The bottom layer of the model E (1:1 1%PVP : blood) had in a large quantity neutrophils because sedimentation agent : blood ratio was suitable. PVP had grouped the blood cells and neutrophils were settled down with erythrocytes. Figure 4 is bottom layer of model E after erythrocyte lysis.

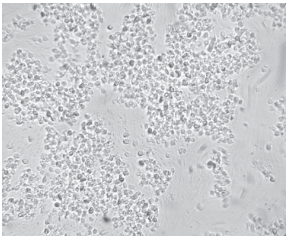

Fig. 4. Neutrophils in bottom layer of model E (1:1 1%PVP : blood) with no dye.

CONCLUSION

Five methods for blood sedimentation were studied. We proved that sedimentation with 1:1 1% PVP : blood was very suitable method for obtaining neutrophils in a large amount. This model ensured of high amount of blood neutrophils and high elastase activity.

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