Original Research Article

Evaluation of effectiveness of different regiments of centrifugation for a single-spin method of pure platelet-containing plasma preparation

ABSTRACT

Aims: The aim of the study is to determine the optimal parameters of single centrifugation for the preparation of platelet-containing plasma (PCP) with maximum reduction of other cellular elements of the blood.

Materials and methods: 30 conditionally healthy persons aged 18 to 60 years (36.9±11.2 years) were included in the study. A total of 12 centrifugation modes were studied: 110g × 5 min, 110g × 10 min, 110g × 15 min, 140g × 5 min, 140g × 10 min, 140g × 15 min, 160g × 5 min, 160g × 10 min, 160g × 15 min, 190g × 5 min, 190g × 10 min, 190g × 15 min. To evaluate the effectiveness of different centrifugation modes, in addition to the number of basic cellular elements, such indicators as platelet capture efficiency, platelet enrichment factor, erythrocyte-reducing efficiencies and leukocyte-reducing efficiencies were studied.

Results: When examining the volumes of the obtained plasma containing platelets, it was found that almost all centrifugation modes allow obtaining significantly different volumes of the investigated blood fraction from the others ($p<0.001$). For clarity, the regimens were sorted according to the volume of platelet-containing plasma obtained, from the smallest to the largest. There was a progressive decrease in the numerical values of the concentration of platelets, erythrocytes and leukocytes in plasma samples. Also there was a progressive decrease in the numerical values of the coefficient of platelet enrichment.

Conclusions: With a single centrifugation for the preparation of plasma containing platelets, the most effective mode is 160g × 10 min, which allows achieving a platelet enrichment factor of about 1.71 at a platelet concentration of 483.6 ± 45.4 × 10^9/l, a platelet capture efficiency of 85, 7 ± 0.1% and reductions of erythrocytes and leukocytes 98.76 ± 0.09% and 98.46 ± 0.14%, respectively.

Keywords: platelet-containing plasma; centrifugation; single-spin method; platelet-rich plasma.

1. INTRODUCTION

According to the American Red Cross, platelet-rich plasma is the fraction of blood that contains platelets in a concentration of at least 5,5 × 10^{10} per 50 ml. [1, 2]. Considering that

the normal number of platelets according to various data varies from 150 to 450 × 10^{10} per liter, the concentration of platelets in the final product should be increased by 2-7 times compared to whole blood.

Currently, in the scientific literature, one can find a huge number of studies aimed at the development and optimization of protocols for the processing of whole blood in order to obtain platelet-rich plasma [1-10]. Among all the diversity of research, there can be found scientific works in which methods of processing blood of both humans and animals have been studied. Studies also differ in the equipment used, with some studies using conventional test tubes, while others used commercial systems. In addition, known protocols for the preparation of platelet-rich plasma are characterized not only by different centrifugation parameters, but also by the multiplicity of centrifugation itself.

The main parameters of the centrifugation mode are relative centrifugal force (RCF) and centrifugation time [11]. The disadvantage of a number of studies is the indication in the centrifugation methodology not of the relative centrifugal force, but of the centrifugation speed, expressed in revolutions per minute. At the same time, the researchers do not indicate the value of the rotation radius of the centrifuge rotor, without which it is impossible to convert the centrifugation speed into relative centrifugal force, and therefore, to use this technique with the use of centrifuges of other models.

It should be noted that the characteristics of the final product also differed in the above studies. Thus, the final product could be positioned as pure or leukocyte-containing plasma enriched with platelets. In some studies, the parameters of the content of other formed elements of blood in the final product were not indicated at all, although this characteristic has a significant impact on the results of the clinical application of plasma enriched with platelets.

The total volume of erythrocytes in whole blood (hematocrit) is 42-52% for men and 37-47% for women [12]. That is why not only its total volume depends on the degree of purification of the final product from erythrocytes, but also the coefficient of its enrichment with platelets in comparison with whole blood.

As for leukocytes, inflammatory mediators are contained in these cellular elements, which are locally released after the clinical use of leukocyte-containing plasma enriched with platelets. The release of pro-inflammatory cytokines activates NF-κB signaling and can initiate or maintain the inflammatory process [13-15]. Such an effect can have positive consequences in the treatment of acute processes in the initial stages, when inflammation is an important stage of the healing process, but is undesirable in the later stages [16].

Such an effect can have positive consequences in the treatment of acute processes in the initial stages, when inflammation is an important stage of the healing process, but is undesirable in the later stages [16]. It was proved that at later times, leukocytes create a negative effect on stem cells, suppressing their proliferation, reducing the levels of growth factors, increasing the concentration of catabolic cytokines and inducing apoptosis [17].

On the other hand, although leukocytes participate in the immune response, their presence or absence in platelet-rich plasma does not affect its antimicrobial properties [18]. Thus, modern evidence suggests that platelets are involved in antimicrobial protection due to their ability to release powerful antimicrobial peptides from their alpha granules [19-21]. In a number of studies, it has been demonstrated that these peptides have broad-spectrum antimicrobial activity against gram-negative, gram-positive, and fungal pathogens.

The presence of a large number of unsystematized literature data, which sometimes contradict each other, prompted us to investigate different modes of single centrifugation in order to prepare highly purified plasma containing platelets from other cellular elements.

The purpose of the study is to determine the optimal parameters of single centrifugation for the preparation of platelet-containing plasma (PCP) with maximum reduction of other cellular elements of the blood.

2. MATERIAL AND METHODS

The study is a fragment of research work “Development and implementation of innovative technologies in the treatment and prevention of bleeding from varicose veins of the esophagus”, state registration number – 0120U101363.

Prospective study was approved by the Committee on Bioethics, National Pirogov Memorial Medical University, Vinnytsya, Vinnytsia, Ukraine. The Bioethics Committee considered that research was performed in accordance with the World Medical Association Declaration of Helsinki on the ethical principles for medical research involving human subjects, the Council of Europe Convention on the Human Rights and Biomedicine, relevant laws, orders of the Ministry of Health of Ukraine. Each subject of the study was provided with all details about medical procedures and given the opportunity to discuss any questions with healthcare professionals, and then signed a detailed form of informed consent to conduct the research.

30 conditionally healthy persons aged 18 to 60 years (36.9±11.2 years) were included in the study. There were equal numbers of women and men in this contingent – 15 each (50%).

The criteria for inclusion in the study were:

1. Consent of the potential participant to participate in the study.
2. Age from 18 to 60 years.
3. Absence at the time of inclusion in the study of urgent conditions.
4. Absence of potential research participants with known chronic diseases, including in a state of stable remission.
5. Compliance by potential participants within 7 days before the start of the study of the proposed drinking regimen with consumption of at least 1.5 liters of liquid per day.
6. In women, the study had to be conducted in the period between at least the 8th day after the end of the last menstruation and the beginning of the next menstrual cycle.
7. Absence of taking medications affecting the qualitative and quantitative characteristics of blood for at least 21 days before the study.

The exclusion criteria from the study were:

1. Refusal to participate in the study.
2. Non-compliance by potential participants within 7 days before the start of the study of the proposed drinking regimen with consumption of at least 1.5 liters of liquid per day.
3. Women whose menstruation began before the due date, and the study fell on the period of menstruation itself or the next 8 days, were excluded from the study.
4. The presence of pathological changes on the part of the blood according to the results of the conducted research.

When planning the study, preference was given to the one-donor model, as it allows to receive more reliable results.

As mentioned earlier, the main parameters of the centrifugation mode are the relative centrifugal force and the centrifugation time.

According to literature data, RCF less than 110g, even with a centrifugation duration of 15 minutes, is ineffective due to the impossibility of qualitative separation of platelets from erythrocytes and leukocytes [5]. At the same time, with an RCF of more than 180g and a centrifugation time of 10 minutes, in the test tubes an intermediate layer (“buffy coat”)

begins to form, containing leukocytes and platelets, which do not separate from each other [5].

In our study, a CM-3M centrifuge (MICROmed, Ukraine) was used with the ability to set the centrifugation speed in revolutions per minute in steps of 100rpm and a rotation radius of the rotor of 10.0 cm.

According to the effective RCF values described in the literature, centrifugation speeds of 1000 rpm (RCF ≈ 110g), 1100 r/min (RCF ≈ 140g), 1200 rpm (RCF ≈ 160g), and 1300 rpm (RCF ≈ 190g). Centrifugation times of 5, 10, and 15 minutes are most often described in the literature.

Recalculation of the centrifugation speed in RCF was carried out according to the formula:

$$RCF = 11.18 \times r \times \left(\frac{RPM}{1000}\right)^2,$$

r – rotation radius of the rotor in centimeters;

RPM – centrifugation speed in rpm.

Thus, a total of 12 centrifugation modes were studied: 110g × 5 min, 110g × 10 min, 110g × 15 min, 140g × 5 min, 140g × 10 min, 140g × 15 min, 160g × 5 min, 160g × 10 min, 160g × 15 min, 190g × 5 min, 190g × 10 min, 190g × 15 min.

To study the characteristics of the plasma containing platelets obtained at different centrifugation modes, 117 ml of blood was collected from each study participant in 13 vacuum tubes of Vacutest® (KIMA, Italy) with a volume of 9 ml each, which contained sodium heparin at the rate of 17 IU per 1 ml of blood (153 IU per tube). Blood sampling was performed from the cubital vein using 21G needles (Ø 0.8 × 25 mm) for taking multiple blood samples in one procedure and vacuum tube holders (KIMA, Italy).

To avoid individual technical errors, all procedures were performed by one specialist.

After blood collection, one tube with whole blood was sent for a complete hematological examination to determine the main hematological parameters for our study (the number of erythrocytes, leukocytes, platelets) and possible detection of hidden diseases manifested by changes in the quantitative and qualitative composition of blood.

All hematological studies were performed on a BC-6000 device (Mindray, USA).

The remaining 12 tubes of blood samples were centrifuged according to the parameters given above. After centrifugation, the contents of the test tube were divided into two or three layers, depending on the centrifugation parameters. Regardless of the number of layers into which the contents of the test tube were divided, only the upper layer, which was plasma containing platelets, was transferred to vacuum tubes using a single-channel pipette dispenser of variable (from 1 to 10 ml) volume (Thermo Scientific, USA) Vacutest® (KIMA, Italy) without filler, 9 ml each. At the same time, the indicators of the dispenser were recorded to determine the volume of the received plasma containing platelets. The test tubes were sent for a hematological examination with the determination of the number of erythrocytes, leukocytes, and platelets in the product obtained at different centrifugation modes.

To evaluate the effectiveness of different centrifugation modes, in addition to the number of basic cellular elements, such indicators as platelet capture efficiency (PCE), platelet enrichment factor (PEF), erythrocyte-reducing efficiencies (ERE, %) and leukocyte-reducing efficiencies (LRE, %) were studied.

These indicators were calculated according to the following formulas.

$$PCE = \frac{V_{PCP} \times C_{Platelet\_in\_PCP}}{V_{Whole\_blood} \times C_{Platelet\_in\_Whole\_blood}};$$

$$PEF = \frac{C_{Platelet\_in\_PCP}}{C_{Platelet\_in\_Whole\_blood}};$$

$$ERE = 100 - \frac{V_{PCP} \times C_{Leucocyte\_in\_PCP}}{V_{Whole\_blood} \times C_{Leucocyte\_in\_Whole\_blood}};$$

$$LRE = 100 - \frac{V_{PCP} \times C_{Erytrocyte\_in\_PCP}}{V_{Whole\_blood} \times C_{Erytrocyte\_in\_Whole\_blood}},$$

V – the volume of the corresponding component specified in the index, ml;
C – the concentration of the corresponding cellular element specified in the index in whole blood or plasma containing platelets, expressed in units SI (Système international d'unités).
The obtained data were processed using a package of statistical programs IBM SPSS 20.0 for Windows. Values were expressed as the Mean ± Standard Deviation. The independent-samples Student's t-test was performed to analyze differences in data between groups. One-way analysis of variance (ANOVA) with Bonferoni-Sidak Corected p-value was performed to analyze the difference in data between different regiments of centrifugation.

3. RESULTS AND DISCUSSION

During the hematological analysis of the studied whole blood samples, the concentration of erythrocytes was 4.453 ± 0.264 × 10^{12}/l, leukocytes – 6.518 ± 0.511 × 10^{9}/l, platelets – 282.6 ± 21.3 × 10^{9}/l, which corresponded to the reference values.
Taking into account that all plasma samples obtained under different centrifugation modes contain platelets in one or another amount, in our opinion, in the future it is appropriate to call this blood fraction "plasma containing platelets".
The volume of plasma containing platelets obtained under different centrifugation modes is shown in Fig. 1. For clarity, the regimens were sorted according to the volume of platelet-containing plasma obtained, from the smallest to the largest. Subsequently, all other studied indicators were sorted in the same order as the centrifugation modes.

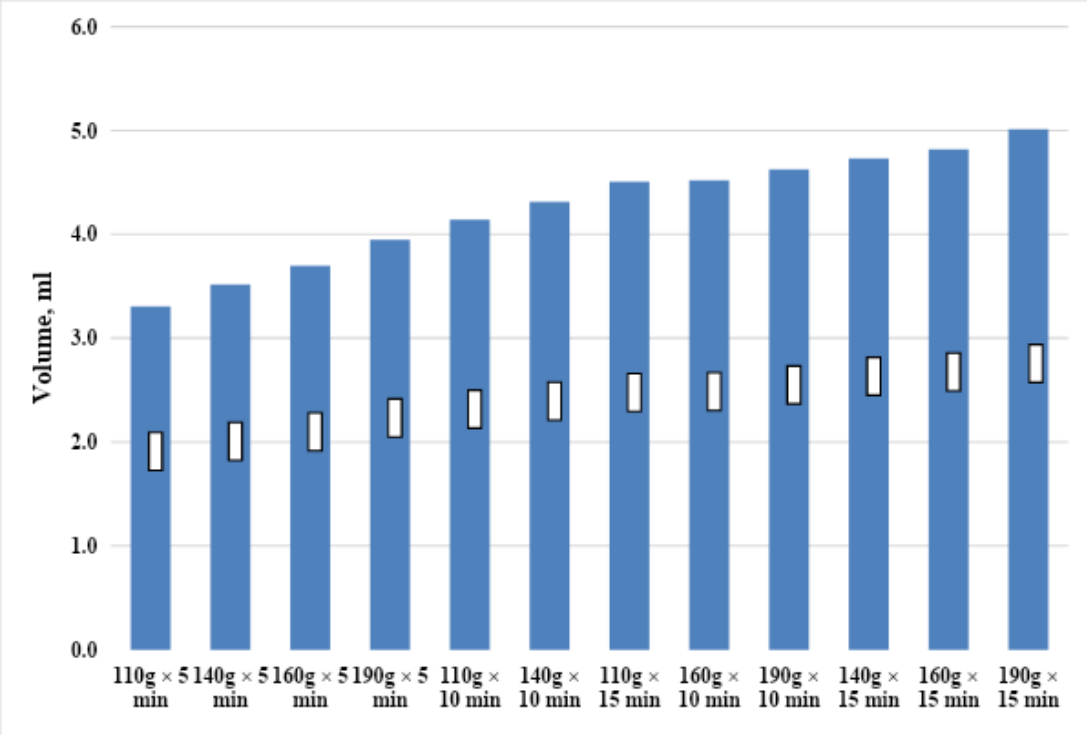

Fig. 1. The volume of plasma containing platelets obtained in different centrifugation modes

When examining the volumes of the obtained plasma containing platelets, it was found that almost all centrifugation modes allow obtaining significantly different volumes of the investigated blood fraction from the others ($p<0.001$). The exceptions were the regimes of 160g × 10 min, 190g × 10 min, 140g × 15 min, and 160g × 15 min, in which the obtained volumes were not significantly different from each other ($p>0.05$).

The concentration of platelets, erythrocytes, and leukocytes in plasma containing platelets obtained at different centrifugation modes is shown in Fig. 2-4, respectively.

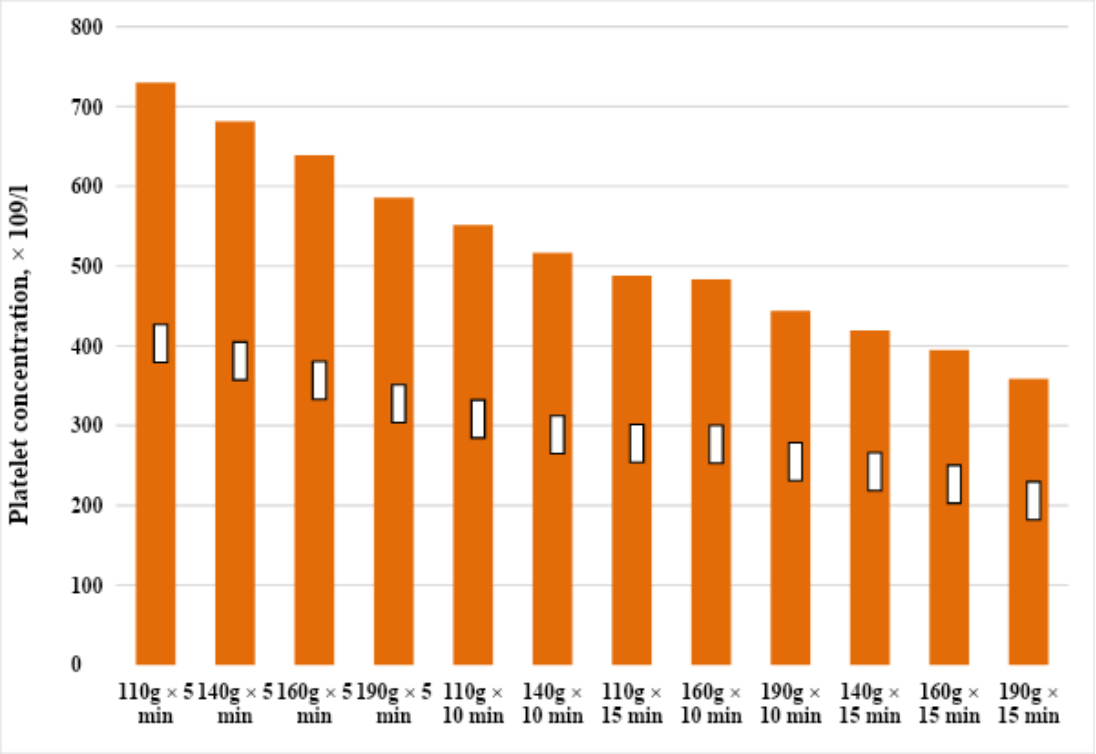

Fig. 2. Concentration of platelets in plasma containing platelets obtained at different centrifugation modes

When studying the concentration of platelets in the obtained samples of the studied blood fraction, a trend opposite to that observed when studying the volumes was observed. Thus, there was a progressive decrease in the numerical values of the studied indicator in plasma samples containing platelets. In addition, it turned out that almost all centrifugation modes allow obtaining platelet concentrations that are significantly different from other concentrations ($p<0.001$). Although the numerical values of the concentration of platelets in the centrifugation mode of 110g × 15 min slightly exceeded the similar indicator in the mode of 160g × 10 min, the statistical significance of the differences could not be confirmed ($p>0.05$).

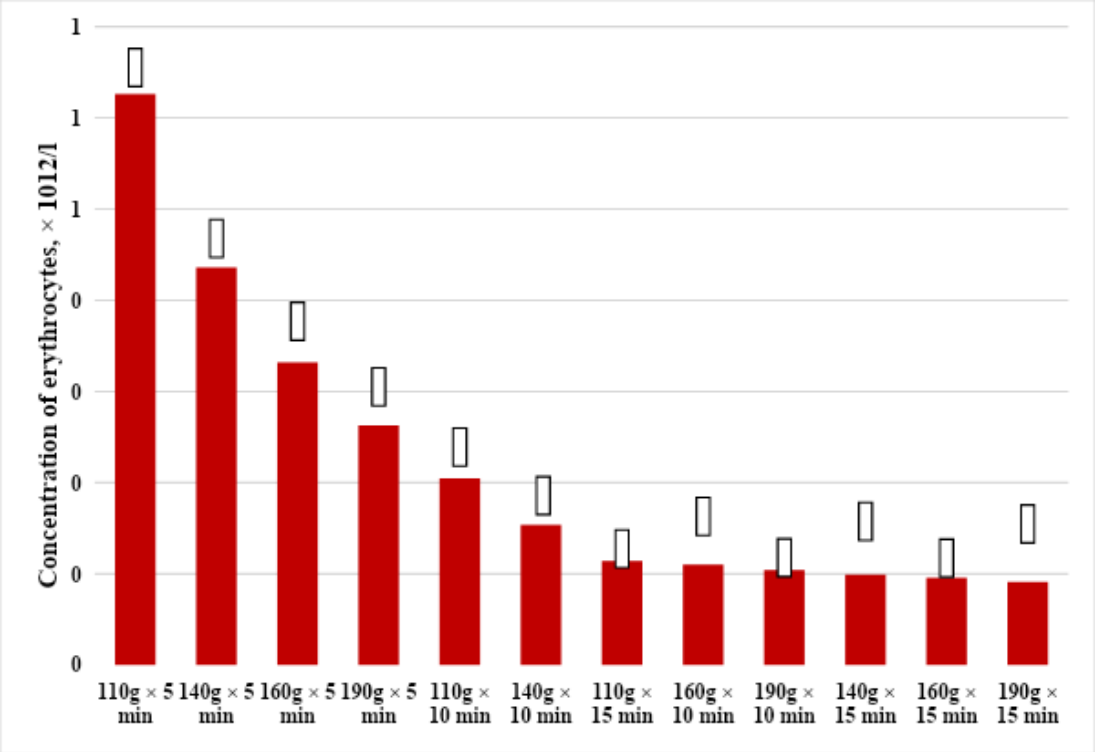

Fig. 3. Concentration of erythrocytes in plasma containing platelets, obtained in different centrifugation modes

When studying the concentration of erythrocytes in the obtained samples of the investigated blood fraction, a trend opposite to that observed when studying the volumes was observed. Thus, there was a progressive decrease in the numerical values of the studied indicator in plasma samples containing platelets. It should be noted that a statistically significant ($p<0.001$) decrease in the concentration of erythrocytes was observed only when comparing the first six regimens. Starting with the 110g × 15 min regimen, statistical significance between adjacent regimens was lost ($p>0.05$).

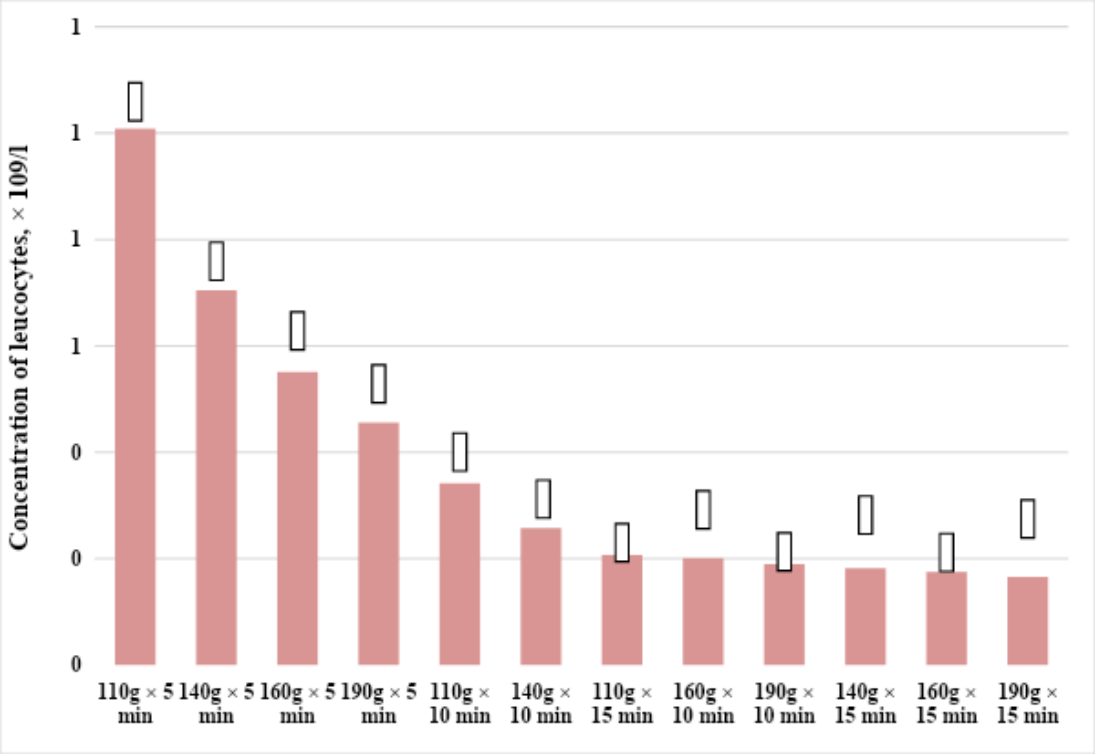

Fig. 4. Concentration of leukocytes in plasma containing platelets, obtained at different centrifugation modes

When examining the concentration of leukocytes in the obtained samples of the studied blood fraction, a trend was observed similar to that observed when examining the concentration of erythrocytes with a statistically significant ($p<0.001$) decrease in the studied indicator when comparing the first six regimens and a loss of statistical significance ($p>0.05$), starting with the regimen 110g × 15 min.

The effectiveness of the reduction of erythrocytes and leukocytes and the capture of platelets in plasma containing platelets obtained at different centrifugation modes is shown in Fig. 5.

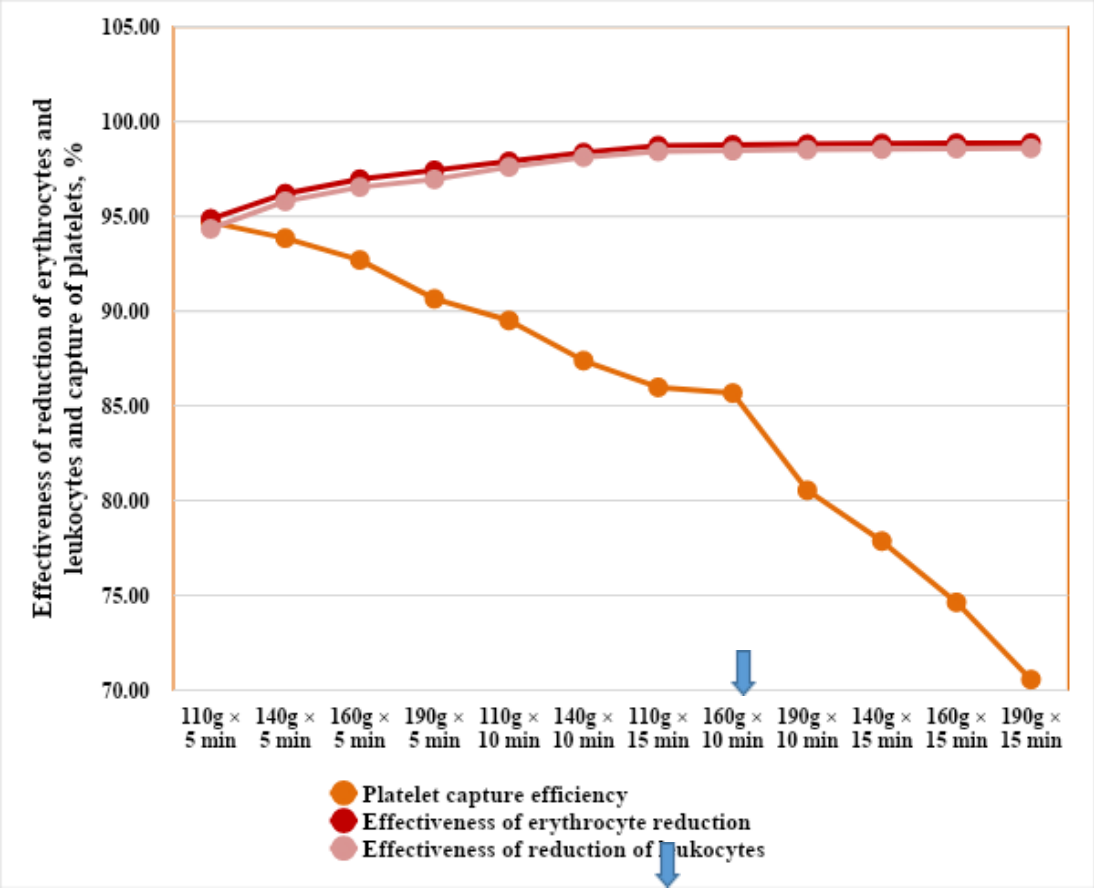

Fig. 5. Effectiveness of reduction of erythrocytes and leukocytes and capture of platelets in plasma containing platelets obtained at different centrifugation modes

Indicators of reduction of erythrocytes and leukocytes had a tendency opposite to the indicators of concentration of these cellular elements. Thus, the numerical indicators of erythrocyte reduction progressively and reliably ($p<0.001$) increased from 94.86 ± 0.09% to 98.35 ± 0.09% when studying the first six regimens. The further increase in numerical values, starting from the regime of 110g × 15 min, ranged from 98.72 ± 0.09% to 98.86 ± 0.09% and was devoid of statistical significance ($p>0.05$). Similarly, the numerical indicators of leukocyte reduction progressively and reliably ($p<0.001$) increased from 94.34 ± 0.14% to 98.12 ± 0.14% when studying the first six regimens, and the subsequent increase in numerical values, starting with the 110g × regimen 15 min, ranged from 98.42 ± 0.14% to 98.59 ± 0.14% and was devoid of statistical significance ($p>0.05$).

As for the efficiency of platelet capture, all centrifugation modes allowed to obtain significantly different values of this indicator in the studied blood fraction ($p<0.001$). The numerical values of this indicator ranged from 94.7 ± 0.1% when using the 110g × 5 min regimen to 70.6 ± 0.1% when using the 190g × 15 min regimen. At the same time, the majority of platelets were lost at centrifugation modes starting from 190g × 10 min. Thus, the regimes from 110g × 5 min to 160g × 10 min were characterized by platelet capture indicators ranging from 94.7 ± 0.1% to 85.7 ± 0.1%. Regimes from 190g × 10 min to 190g × 15 min were characterized by platelet capture indicators ranging from 80.5 ± 0.1% to 70.6 ± 0.1%.

The coefficient of platelet enrichment in different centrifugation modes is shown in Fig. 6.

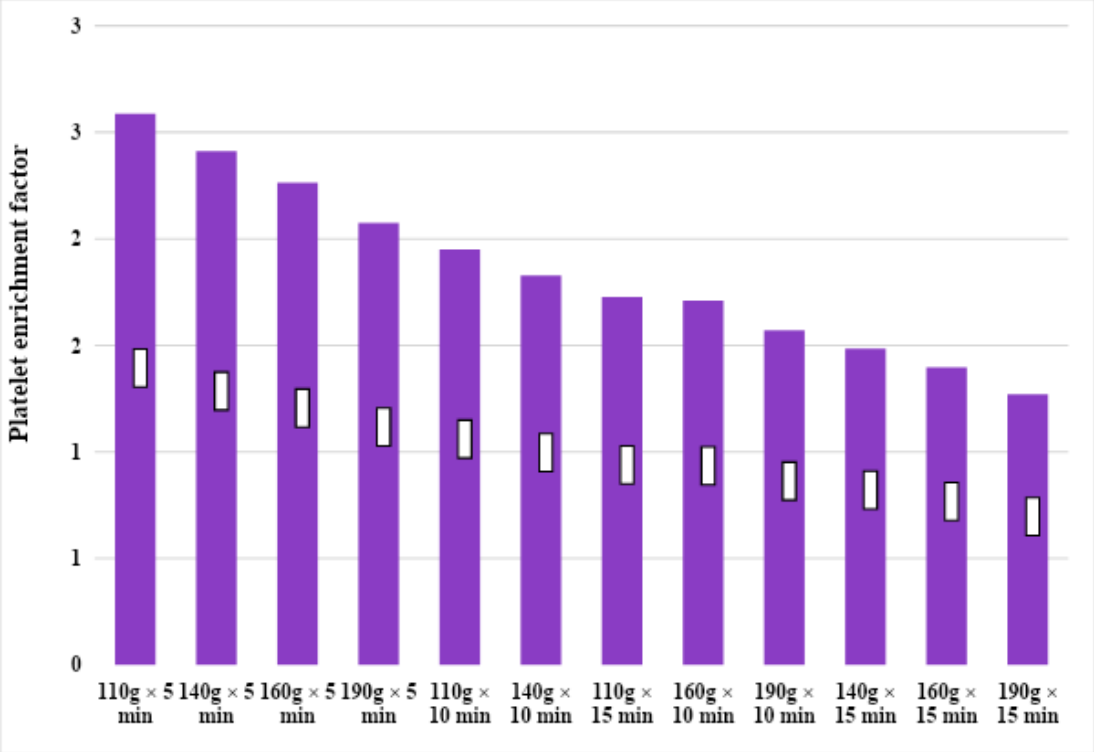

Fig. 6. Platelet enrichment factor at different centrifugation modes

When studying the coefficient of platelet enrichment in the obtained samples of the studied blood fraction, a trend opposite to that observed when studying volumes was observed. Thus, there was a progressive decrease in the numerical values of the studied indicator in plasma samples containing platelets. In addition, it turned out that almost all centrifugation modes allow obtaining significantly different platelet enrichment coefficients from others ($p<0.001$). The exceptions were the regimes of 110g × 15 min and 160g × 10 min, in which the numerical values of the studied indicator, although they were excellent in favor of the first regime, but without reaching the level of statistical reliability of these differences ($p>0.05$).

As demonstrated in our study, both an increase in relative centrifugal force and an increase in centrifugation time resulted in an increase in the platelet-containing plasma volume obtained. This tendency is fully and completely explained by the theory of centrifugation [11].

As noted earlier, for clarity, the centrifugation modes were sorted according to the volume of platelet-containing plasma obtained, from smallest to largest. Subsequently, all other studied indicators were sorted in the same order as the centrifugation modes.

Upon further analysis, it was found that such studied indicators as the number of platelets, erythrocytes and leukocytes, as well as the efficiency of platelet capture and the coefficient of platelet enrichment had a trend opposite to that in the sorting regimes when examining volumes of plasma containing platelets. That is, when using each subsequent centrifugation

regime, in which the volume of plasma containing platelets increased, the values of the studied indicators decreased.

The opposite trend occurred when studying the effectiveness of red blood cell and leukocyte reduction, when the use of each subsequent centrifugation regime, in which the volume of plasma containing platelets increased, the values of the studied parameters also increased.

If high rates of erythrocyte reduction were achieved due to the selection of centrifugation modes, then leukocyte reduction, in our opinion, was achieved mainly due to the fact that we selected only the upper layer of plasma for the study. During the formation of the intermediate layer ("buffy coat"), which contains leukocytes and platelets [5, 22], it has been neglected. At the same time, although a certain amount of platelets was lost, it was possible to achieve high levels of leukocyte reduction.

When analyzing the obtained data, it was demonstrated that the regimes of 110g × 15 min to 160g × 10 min did not statistically significantly differ from each other in almost all the studied parameters. In addition, it should be noted that, starting with these two regimes, the indicators of red blood cell and leukocyte reduction, although there was a further increase in numerical values, however, it was devoid of statistical reliability. At the same time, the majority of platelets were lost in centrifugation regimes, immediately after the 160g × 10 min regime, starting with 190g × 10 min, which indicates a faster reduction of platelets with an increase in the relative centrifugal force and duration of centrifugation.

In our opinion, despite the statistically implausible advantages of plasma containing platelets obtained with the 160g × 10 min regimen over the 110g × 15 min regimen, the main argument in favor of the former is the shorter centrifugation time.

That is why, in our opinion, when using ordinary test tubes, centrifugation in the mode of 160g × 10 min allows obtaining the most qualitatively purified plasma containing platelets from other formed elements. Although this mode of centrifugation does not allow achieving the minimum two-fold concentration of platelets, which is a condition for characterizing the obtained product as plasma enriched with platelets, this shortcoming can easily be compensated by the second centrifugation.

Among all studies, only the study by Wenjing Yin et al showed a similar result [5]. This study was also supported by data on the benefits of this centrifugation regimen by studies of levels of growth factors contained in platelets, as well as levels of pro-inflammatory cytokines.

4. Conclusion

With a single centrifugation for the preparation of plasma containing platelets, the most effective mode is 160g × 10 min, which allows achieving a platelet enrichment factor of about 1.71 at a platelet concentration of $483.6 \pm 45.4 \times 10^9$/l, a platelet capture efficiency of 85, 7 ± 0.1% and reductions of erythrocytes and leukocytes 98.76 ± 0.09% and 98.46 ± 0.14%, respectively.

Ethical approval (where ever applicable)

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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