The blood cells count in leukocyte and leukocyte-poor platelet-concentrated plasma in patients with musculoskeletal disorders

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ABSTRACT

The cellular composition significantly affects the properties of platelet concentrates. In particular, leukocyte platelet concentrates due to the increased number of monocytes and granulocytes have increased levels of proinflammatory cytokines, which contribute to the destruction of extracellular matrix, reduce synthesis of its components and enhance inflammation in tissues.

THE PURPOSE. To determine the blood cells number in leukocyte (L-PCP) or leukocyte-poor platelet-concentrated plasma (LP-PCP) and compare it with whole venous blood, plasma and platelet-poor plasma.

MATERIALS AND METHODS. 20 L-PCP and 21 LP-PCP samples were obtained by double centrifugation from the venous blood of 30 donors aged 26 to 78 with local pathology of the musculoskeletal system. In the first stage, plasma was isolated after the separation of whole blood, and in the second stage, platelets were concentrated in a small volume of platelet-poor plasma. The number of blood cells in the test samples was determined by a hematology analyzer.

RESULTS. When comparing the platelet count in L-PCP and LP-PCP, no significant difference was found in platelet number ($2803.2 \pm 933.8 \cdot 10^6$ and $2260.0 \pm 943.9 \cdot 10^6$ cells/mL, respectively). The efficiency of platelet concentration from the initial volume of whole blood for the methods of obtaining LP-PCP and L-PCP did not differ significantly and was $76.5 \pm 23.4 \%$ and $83.0 \pm 24.9 \%$, respectively. Platelet count in L-PCP was 8, 13 and 239 times higher than in plasma, whole blood and platelet-poor plasma, respectively. The number of platelets in LP-PCP while exceeded their count in the same samples by 8, 11 and 194 times, respectively. The leukocyte number in leukocyte-poor and leukocyte plasma with platelet concentrate was $2.5 \pm 1.2 \cdot 10^6$ and $5.9 \pm 1.8 \cdot 10^6$ cells/mL, respectively. However, their number in LP-PCP was almost 2 times lower than in blood and 5 times higher compared with plasma. The number of leukocytes in L-PCP was 30 % higher than in the blood, and 6 times higher than in plasma. The erythrocyte number was reduced by 99 % in both types of plasma with platelet concentrate compared with their count in whole blood.

CONCLUSIONS. According to the parameter of platelet isolation from whole blood, the procedures for obtaining leukocyte or leukocyte-poor platelet-concentrated plasma belong to the methods with medium efficiency. The procedure for preparing L-PCP allows to get a product with an increased relative number of leukocytes. It was possible to achieve a significant reduction in erythrocyte count for both studied products.

KEY WORDS: platelet concentrates; platelet-rich plasma; platelet-concentrated plasma

Platelet concentrates are biotechnological blood products with a platelet count greater than the normal value, which is $150-450 \cdot 10^6$ cells/mL. Platelet-rich plasma (PRP) refers to liquid platelet concentrates [17, 27, 31]. Various blood processing methods are used to produce PRP, which mainly include centrifugation [17]. Different centrifugal force and the number of centrifugations significantly affect the cell composition of the obtained PRP [34]. In particular, during double centrifugation, a larger number of platelets is sedimented [5], and to obtain leukocyte-poor PRP, one centrifugation is preferably used [26, 32]. At one centrifugation it is usually

possible to increase the concentration of thrombocytes no more than in 3 times in comparison with whole blood. At present, many methods for obtaining PRP have been developed and numerous commercial kits have been designed for its preparation with different platelet isolation efficiency [5, 6, 17]. Platelet-concentrated plasma (PCP) is also obtained from PRP by platelet sedimentation and 90 % plasma elimination [5].

PRP is used in many fields of medicine, such as orthopedics [31], surgery [2, 19], sports medicine [21], dentistry [4], gynecology [14], cosmetology [18] and others. The optimal concentration of platelets in

PATIENT	AGE	SEX	DISEASE	PCP TYPE
1	61	Man	Gonarthrosis	L-PCP
				LP-PCP
2	78	Woman	Shoulder osteoarthritis	LP-PCP
3	74	Woman	Gonarthrosis	L-PCP
				LP-PCP
4	36	Woman	Delayed fracture healing	L-PCP
5	59	Woman	Gonarthrosis	L-PCP
				LP-PCP
6	46	Woman	Supraspinatus tendon injury	LP-PCP
7	66	Woman	Gonarthrosis	L-PCP
				LP-PCP
8	63	Man	Meniscus tear	L-PCP
				LP-PCP
9	57	Woman	Coxarthrosis	L-PCP
10	56	Woman	Gonarthrosis	L-PCP
				LP-PCP
11	36	Man	Tibial unconsolidated fracture	L-PCP
12	33	Man	Osteochondrosis	LP-PCP
13	29	Man	Osteochondrosis	L-PCP
14	69	Man	Ankle joint arthrosis	LP-PCP
15	48	Man	Osteochondrosis	LP-PCP
16	62	Woman	Gonarthrosis	L-PCP
				LP-PCP
17	55	Woman	Coxarthrosis	L-PCP
				LP-PCP
18	26	Woman	Chondromalacia patellae	L-PCP
				LP-PCP
19	48	Woman	Coxarthrosis	L-PCP
				LP-PCP
20	60	Woman	Meniscus tear	LP-PCP
21	52	Woman	Gonarthrosis	L-PCP
				LP-PCP
22	57	Woman	Coxarthrosis	L-PCP
23	48	Woman	Coxarthrosis	L-PCP
24	54	Woman	Supraspinatus tendinopathy	L-PCP
25	54	Man	Coxarthrosis	L-PCP
26	58	Man	Gonarthrosis	LP-PCP
27	49	Woman	Meniscus tear	LP-PCP
28	64	Woman	Coxarthrosis	L-PCP
29	28	Man	Meniscus tear	L-PCP
30	49	Woman	Meniscus tear	LP-PCP

Note: L-PCP – leukocyte platelet-concentrated plasma, LP-PCP – leukocyte-poor platelet-concentrated plasma.

PRP to ensure a therapeutic effect is the subject of scientific discussion and numerous studies, which results are ambiguous. It is believed that for PRP the platelet count should be at least $1 \cdot 10^9$ cells/mL to effectively ensure tissue repair [14, 25]. Thus, in tenocyte culture, it was demonstrated that the concentration of platelets from $5 \cdot 10^5$ to $1 \cdot 10^6$ platelets/ μ L in PRP stimulated cell proliferation and migration, as well as collagen synthesis [25]. While the number of platelets from 1 to $1.5 \cdot 10^6$ platelets/ μ L enhances the proliferation of endothelial cells and fibroblasts [26, 27]. On the other hand, high platelet concentrations can lead to inhibition of cell metabolism and reduction of collagen synthesis [8, 25]. However, in the elderly, due to the increase in the concentration of platelets in the PRP, cell proliferation increases [7].

Platelet concentrates also contain other types of circulating blood cells. The number of leukocytes is one of the most important indicators for such products, as they can significantly affect its properties [37, 38]. According to their content, platelet concentrates are divided into leukocyte and leukocyte-poor ones [17]. Leukocyte platelet concentrates are characterized by higher leukocyte count compared to whole blood, while in leukocyte-poor ones their count is lower [28, 46, 50]. Leukocyte platelet concentrates contain significant number of granulocytes and monocytes, which produce proinflammatory cytokines: tumor necrosis factor α (TNF α), interleukins 1 β , 6 and 8. These cytokines induce the expression of genes that promote the destruction of the extracellular matrix and enhance inflammation, including genes of proinflammatory interleukins, prostaglandins, inducible NO-synthase, cyclooxygenase 2 and metalloproteinases [40, 48, 49]. Despite a number of possible negative effects, leukocyte platelet concentrates can promote healing and regulate inflammation, in particular enhance the synthesis of growth factors and have a pronounced antimicrobial effect, so they should not be completely excluded from clinical application [17]. There is also evidence that leukocyte PRP stimulates the proliferation of chondrocytes, tendocytes and myocytes, and, therefore, leukocyte platelet concentrates can be used to treat cartilage disorders, including osteoarthritis [1, 21, 30].

While studying the cellular components of platelet concentrates, insufficient attention is paid to the number of erythrocytes [16]. Some studies have shown that the number of red blood cells in PRP is minor because their count is reduced by 90 % compared with whole blood [9, 40]. However, erythrocytes can be harmful to cells due to increased oxidative damage and affect the synthesis of extracellular matrix [23, 24].

The **PURPOSE** of the study was to compare the blood cells count in leukocyte (L-PCP) and leukocyte-poor platelet-concentrated plasma (LP-PCP), as well as to compare them with venous blood, plasma and platelet-poor plasma.

MATERIALS AND METHODS

The material for the study was blood, plasma and autologous biotechnological products of blood of 30 patients with various diseases and injuries of the musculoskeletal system. There were 20 women aged 26 to 78 years (mean age 55 ± 12 years) and 10 men aged 28-69 years (mean age 48 ± 14 years).

The venous blood was obtained fasting by the informed consent. The 20 leukocyte and 21 leukocyte-poor platelet concentrates were prepared. Platelet-concentrated plasma products were processed by Araki technique [5] with some modifications. 41 control samples of venous blood and plasma were also obtained.

Thrombocytopenia or anaemia was not diagnosed in the study participants. They also had local pathology of the musculoskeletal system without exacerbation of concomitant diseases, so this group can be considered homogeneous. One week before blood sampling, patients were discontinued non-steroidal anti-inflammatory drugs, prescribed a spe-

Table 1. Types of blood products in patients with pathology of the musculoskeletal system.

Fig. 1. The scheme for obtaining leukocyte and leukocyte-poor platelet-concentrated plasma from whole blood (according to Liu F, 2019).

Fig. 2. Platelet number in the samples of LP-PCP and L-PCP in patients with musculoskeletal disorders, $M \pm m$ ($n = 21$).

Note: * – $p \leq 0.05$ compared with L-PCP group.

cial diet (exclusion of fatty, fried and spicy foods, total daily intake up to 3 liters, no coffee) and regimen (prohibition of smoking and drinking alcohol). The distribution of patients by types of blood products and by diseases is presented in **Table 1**.

To prepare the product of leukocyte platelet-concentrated plasma, 50 mL of venous blood was collected in 10 mL Vacutainer™ Blood Collection Tubes with Acid Citrate Dextrose (BD, USA). After sampling, venous blood was centrifuged at 250 xg for 10 min to separate plasma and blood cells using a CM-3 centrifuge (MICROMed, China). After centrifugation, the blood plasma was gently agitated to increase the concentration of leukocytes, as white blood cells rise into the plasma from the upper buffy coat layer. After that, 25–30 mL blood plasma with a high number of leukocytes was transferred into new 10 mL tubes and centrifuged at 2300 xg for 5 minutes. The resulting platelet pellet was resuspended in 3 mL of platelet-poor plasma, which remained when most platelets were removed after the second centrifugation. Since 3 mL of plasma is an optimal volume for injection into the patient's joint to reduce pain from excess injected fluid,

it was chosen for the study. Most volume (about 90 %) of platelet-poor plasma was removed.

To prepare the product of leukocyte-poor platelet-concentrated plasma, 50 mL of venous blood was collected in 10 mL Vacutainer™ Blood Collection Tubes with Acid Citrate Dextrose (BD Vacutainer, USA). Blood plasma after the first centrifugation at 250 xg for 10 min was collected in new 10 mL vials without agitation to reduce the number of leukocytes. After that, the collected plasma was centrifuged at 2300 xg for 5 minutes. The resulting cell pellet was also resuspended in 3 mL of platelet-poor plasma. Most volume of the platelet-poor plasma was removed. The general scheme for obtaining PCP is shown in Fig. 1.

To study the cell count in whole blood, plasma and platelet-poor plasma, samples of about 1.5 mL were taken. Platelet-concentrated plasma was diluted 2 times with Hartmann's solution (Infusion, Ukraine) prior to cells number count. Blood, plasma, L-PCP and LP-PCP samples were analyzed on a Celltac Alpha hematology analyzer (Nihon Kohden, Japan).

CELL TYPE	WHOLE BLOOD	PLASMA	LP-PCP	PLATELET-POOR PLASMA
White blood cells, 10^6 cells/mL	4.5 ± 1.2	$0.5 \pm 0.2^*$	$2.5 \pm 1.1^*$	$0.1 \pm 0.0^*$
Red blood cells, 10^6 cells/mL	4040.0 ± 385.3	$13.0 \pm 5.71^*$	$43.0 \pm 16.7^*$	$2.63 \pm 4.5^*$
Platelets, 10^6 cells/mL	191.0 ± 42.1	$279.05 \pm 75.6^*$	$2260.0 \pm 943.9^*$	$11.9 \pm 8.5^*$

Table 2. The number of different cell types in blood, plasma, leukocyte-poor platelet-concentrated plasma (LP-PCP) and platelet-poor plasma; $n = 21$

Note: * – $p \leq 0.05$ compared with whole blood.

CELL TYPE	WHOLE BLOOD	PLASMA	L-PCP	PLATELET-POOR PLASMA
White blood cells, 10^6 cells/mL	4.7 ± 0.7	$1.0 \pm 0.3^*$	$5.9 \pm 1.8^*$	0.095 ± 0.0
Red blood cells, 10^6 cells/mL	4012.0 ± 417.6	$15.2 \pm 6.0^*$	$50.0 \pm 2.0^*$	$1.42 \pm 3.6^*$
Platelets, 10^6 cells/mL	208.8 ± 36.7	$336.8 \pm 70.4^*$	$2803.0 \pm 933.8^*$	$11.71 \pm 7.6^*$

Table 3. The number of different cell types in blood, plasma, leukocyte platelet-concentrated plasma (L-PCP) and platelet-poor plasma ($n = 20$).

Note: * – $p \leq 0.05$ compared with whole blood.

Numerical characteristics of the parameters were calculated by the methods of descriptive statistics and are presented in the form of average value and standard deviation $M \pm m$. Student's t-test with Bonferroni correction was used as a criterion for the reliability of differences between the compared groups. P-values of less than 0.05 were regarded as statistically significant. Statistical analysis of data was performed using MS Excel software (Microsoft, USA).

RESULTS AND DISCUSSION

The study of peripheral blood cell count of 30 patients found that the number of leukocytes, erythrocytes and platelets was $4.6 \pm 1 \cdot 10^6$ cells/mL, $4014.0 \pm 397.7 \cdot 10^9$ cells/mL and $196.6 \pm 43 \cdot 10^6$ cells/mL, respectively.

The number of platelets in LP-PCP and L-PCP was $2260.0 \pm 943.9 \cdot 10^6$ and $2803.2 \pm 933.8 \cdot 10^6$ cells/mL, respectively (Fig. 2 A). Therefore, no significant difference was found between the platelet count in these biotechnological products, which coincides with the data of some studies on platelet-rich plasma [36, 40, 43, 47]. However, some studies have shown that the number of platelets in leukocyte-poor platelet concentrates is significantly lower than in leukocyte-containing ones [6, 39]. Since many methods of LP-PCP preparation use single centrifugation, it significantly reduces the number of platelets compared with L-PCP [6, 34, 38].

The efficiency of platelet isolation for both methods of obtaining concentrates is also comparable and is $76.5 \pm 23.4\%$ and $83.0 \pm 24.9\%$ for LP-PCP and L-PCP, respectively. Therefore, the protocols used for obtaining LP-PCP and L-PCP belong to the methods with average efficiency of platelet concentration from whole blood according to DEPA classification of such cellular products proposed by Magalon J. [28].

It was shown that the number of platelets in LP-PCP was increased compared with plasma, whole blood and platelet-poor plasma in 8, 11 and 194 times, respectively (Table 2). While the number of platelets in L-PCP exceeded their count in leukocyte-containing plasma, whole blood and platelet-poor plasma in 8, 13 and 239 times, respectively (Table 3). There is a lack of literature data on the influence of many factors, such as age and concomitant pathology, on the platelets number in cell concentrate products. However, Weibrich G. [44] showed that age has almost no effect on platelet counts in PRP. In addition, Pauly S. [38], who used the blood of patients with tendopathies to prepare PRP, are comparable to data from another study [35] that used blood from healthy individuals. Both studies used the same commercial blood processing technique.

Since the number of platelets in the whole blood of healthy adults varies in a relatively wide range of $150\text{--}450 \cdot 10^6$ cells/mL, this factor must be taken into account in the production of all types of platelet concentrates [16]. In addition, in some diseases, including immune thrombocytopenia and chronic leukocyte leukemia, the platelet number in the blood is reduced to $100 \cdot 10^6$ cells/mL and less [41, 42]. When studying the

Fig. 3. Platelet count in L-PCP and LP-PCP from patients with different number of platelets in whole blood ($M \pm m$; $n = 7\text{--}10$).

Fig. 4. The number of leukocytes in the samples of LP-PCP and L-PCP in patients with musculoskeletal disorders, $M \pm m$ ($n = 21$).

Note: * – $p \leq 0.05$ compared with the group L-PCP.

correlation between the platelets count in whole blood and in the prepared concentrates, the products were divided into two groups according to the number of platelets in the processed blood. It was shown that in L-PCP made from the individuals with the number of platelets in whole blood $150\text{--}200 \cdot 10^6$ cells/mL and $200\text{--}250 \cdot 10^6$ cells/mL, the platelet count was 2586 ± 715 and 3043 ± 999 cells/mL, respectively. While in LP-PCP the number of thrombocytes for the same comparison groups was 2423 ± 613 and 2107 ± 705 cells/mL, respectively (Fig. 3). Therefore, there was no significant difference in platelet count in PCP from patients with increased (more than $200 \cdot 10^6$ cells/mL) and reduced (up to $200 \cdot 10^6$ cells/mL) platelet number in whole blood. An increased number of platelets in the blood above $300 \cdot 10^6$ cells/mL may have a more significant effect on their final count in PCP. However, there were not enough people with high platelet number in our group to study this.

Some authors have shown that the platelets count correlates with the level of growth factors in platelet concentrates [5, 19], in particular, transforming growth factor $\beta 1$, platelet growth factor-BB, hepatocyte growth factor and epidermal growth factor [22, 40].

The number of leukocytes in LP-PCP was reduced by 2 times compared with L-PCP (Fig. 4). It was also 2 times lower than in blood, but 5 times higher than in plasma. This correlates with the data of some other studies [35]. The number of leukocytes in LP-PCP and L-PCP was $2.5 \pm 1.2 \cdot 10^6$ and $5.9 \pm 1.8 \cdot 10^6$ cells/mL, respectively. The leukocytes count in L-PCP was 30 % higher than in the blood, and 6 times higher than in plasma. However, the composition and number of different cell types in platelet concentrates are significantly influenced by different initial conditions in the product processing, including the volume of collected blood, the centrifugal force at blood separation, the number of centrifugations, etc.

It is known that L-PCP products have increased level of pro-inflammatory interleukins. In particular, in the culture of synovial membrane fibroblasts, it was shown that L-PCP enhanced the synthesis of proin-

flammatory IL-1 β and IL-8 [6, 38, 45, 46]. In addition, it was shown that leukocyte-poor platelet-rich plasma significantly enhanced the proliferation of human osteocytes, myocytes and ligament cells [32]. While leukocytes-containing platelet-rich plasma inhibited the proliferation of tenocytes and mesenchymal stem cells, as well as cell differentiation into cartilage and bone tissue [45, 48, 50, 51]. In addition, leukocyte platelet concentrates can reduce the synthesis of extracellular matrix molecules in tissues. In particular, in the cartilage tissue under the influence of IL-1 β and TNF α , the synthesis of the main components of the extracellular matrix (aggrecans and type II collagen) decreases [9, 11]. This can be explained by the production of pro-inflammatory cytokines in leukocyte platelet concentrates, which contribute to catabolic processes in tissues [6]. Despite these negative effects, the use of L-PCP can promote healing processes, in particular to enhance the synthesis of growth factors. In addition, they have a pronounced antimicrobial effect, so they should not be completely excluded from clinical application [17]. Moreover, in clinics there is a lack of data on the negative effects of leukocyte platelet concentrates compared to leukocyte-poor ones [11, 34]. In addition, some clinical studies have shown that platelet-rich plasma with leukocytes did not increase the level of pro-inflammatory interleukins in the synovial membrane of the joint at the treatment of gonarthrosis [29].

The number of erythrocytes was reduced by 99 % in LP-PCP and L-PCP compared with their count in the whole blood. However, red blood cells number in LP-PCP was 3 times higher than in plasma and 18 times higher than in platelet-poor plasma. The number of erythrocytes in L-PCP was 4 and 40 times higher than in native plasma and platelet-poor plasma, respectively. However, erythrocytes can be harmful to cells and tissues [9, 23, 24]. In particular, it has been shown that red blood cells decrease the viability of synoviocytes [9], as well as reduce the synthesis of cartilage proteoglycans [23, 24]. It is assumed that the mechanism of adverse effects of red blood cells is associated with increased oxidative damage caused by iron in hemoglobin [23].

CONCLUSION

Thus, the efficiency of platelet isolation in the procedures of obtaining LP-PCP and L-PCP in patients with musculoskeletal disorder is comparable and they can be attributed to methods with average efficiency of the concentration of platelets from whole blood. There was no significant difference in platelet count in manufactured products from patients with high and low primary platelet count in whole blood. The processing of L-PCP allows to obtain a product with a relative number of leukocytes, which is 30 % higher than in whole blood, and almost twice as much as in LP-PCP. For both studied products it was possible to achieve a significant reduction in the number of erythrocytes compared to its count in the whole blood.

The justification for a differentiated approach to the choice of biotechnological product depending on the pathology of the musculoskeletal system is considered to be the prospect of further research in the clinical application of different types of autologous platelet concentrates.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.