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Technologies for producing platelet masses for regenerative medicine
(оглядова стаття)

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The development of regenerative medicine is to improve existing and to search for new tools for morphological and functional tissue repair, among which plasma or fibrin enriched with platelets (PRP and PRF) can be significant. Autogenic platelet masses stimulate collagen synthesis, induce vascular growth, reduce pain, provide hemostasis, accelerate regeneration, reduce the risk of postoperative infectious and inflammatory complications, and also have powerful osteoinductive properties.

Due to the ability to produce the majority of growth factors, platelets can affect all stages of the inflammatory-regenerative process, and therefore their biological products are of great importance in solving the problems of regenerative medicine. The technologies for obtaining PRP and PRF are based on centrifugation of blood, as a result of which its active components are concentrated in certain areas of the centrifuge.

Blood sampling with or without an anticoagulant, as well as modification of centrifugation protocols, allows to obtain various forms of platelet masses, such as a liquid, gel or clots. They are classified, depending on the cellular content and architecture of fibrin, into several categories, namely: pure plasma enriched in platelets (P-PRP), plasma enriched in leukocytes and platelets (L-PRP); injectable fibrin enriched with platelets (i-PRF) and pure fibrin enriched with platelets (P-PRF), as well as fibrin enriched with white blood cells and platelets (L-PRF).

The main difference in the manufacture of PRP compared to PRF is the use of anticoagulants and activators, as well as the possibility of using two-stage centrifugation.

Platelet mass is used as an independent component mainly to stimulate the restoration of muscle tissue, to heal chronic wounds, to treat articular pathologies, and in combination with other materials, in particular to replace bone defects.

The mechanisms of influence of each of the categories of platelet mass on tissue regeneration remains poorly understood. It is necessary to standardize the protocols for their preparation, taking into account the influence of additional substances, such as platelet activators or blood clotting and anticoagulants, as well as optimization of the methods for using each of the platelet mass forms.

Key words: platelets, PRP, PRF, centrifuges, centrifugal force.

Problem statement, analysis of recent research. In recent years, regenerative biomedicine has acquired significant theoretical and practical development, which represents scientifically based concepts, methods and technologies for the obtaining and storage of cellular and tissue-engineered products, restoration and controlled regeneration of tissues and organs, their structures and functions. The problems of regenerative biomedicine are extremely wide. On the one hand, it is caused by significant differences in the regenerative properties of tissues and organs, and on the other hand, by the loss of their reparative potential [1, 2].

The reasons for this can be various factors, in particular tissue degeneration, oxidative damage and an imbalance of regenerative mechanisms based on the loss of blood supply or large-volume injuries, their complications by infectious and inflammatory processes, metabolic syndrome or

immunopathological processes leading to tissue destruction or metaplasia [3].

The technologies of regenerative medicine turned out to be especially widespread in traumatology and orthopedics for damage to ligaments and tendons, cartilage and bones [1, 4]. Although bone tissue, due to its exclusively cellular type of regeneration, has the unique property of forming identical bone regenerate, the reparative osteogenesis is often complicated by various pathological conditions. However, many innovative approaches have not improved yet the clinical results of treatment of significant bone defects [5, 6]. They are primarily aimed at the use of auto-transplants, allotransplants and tissue-engineering structures associated with cells or other biological factors [1]. Each of the options has certain disadvantages, such as reduced bioactivity of implants, immunological inflammatory reactions, the need for additional surgical intervention, limited availability, inadequate size and shape, as well as transmission of diseases from the donor [7, 8, 9].

This necessitates the improvement and search for new accessible, safe and optimal materials that must meet certain requirements [10]: biocompatibility, stimulation of angiogenesis [5], osteo-conductivity and osteo-inductivity [11], as well as the absence of inflammatory, allergic and toxic reactions [10].

At the same time, significant attention is paid to various growth factors and bone morphogenetic proteins [12, 13]. However, platelet masses can be their alternative; they can enhance and optimize regeneration processes due to the content of all known growth factors in platelets [4, 9, 14].

Platelets and platelet mass.

Platelets are nuclear-free spherical cells with a diameter of 2-4 microns [15]. In the blood stream, they circulate for about 9–11 days, capable of instant adhesion, aggregation, and secretion of their granules contents [16], the first to accumulate in large numbers in areas of damaged vessel and surrounding tissues [17]. These cells contain almost all possible sources of reactive oxygen species, such as xanthine oxidase, cytoplasmic NAD (F) H oxidase, mitochondria, and enzymes that catalyze the conversion of arachidonic acid. Active forms of oxygen perform many functions in the body: participation in the reactions of oxidative phosphorylation, biosynthesis of prostaglandins and nucleic acids, in the processes of mitosis and decay of phagocytized bacterial cells [18].

In platelets there are about 35 α-granules and 5 dense bodies that serve as the main storage tanks for various biologically active substances [17].

Platelet activation occurs by a number of stimulants (thrombin, calcium chloride, collagen, etc.) [19] due to contact with specific receptors located on their surface, or as a result of interaction with collagen, von Willebrand factor and other adhesive proteins. At the same time, the intracellular concentration of calcium ions increases, and as a result, the proteins of the platelets cell membrane mediate adhesion and aggregation [15, 20, 21].

In general, due to its complex organization, platelets carry out and control adhesion, aggregation and primary vascular-platelet hemostasis, angiotrophic and reparative functions, which became the basis for the use of concentrates or a combination with fibrin and leukocytes for a variety of pathologies.

The concept of “platelet masses” is a term that summarizes the name of products made by centrifuging blood immediately after sampling it. They can be activated, not activated, with or without leukocytes, but all are characterized by increased platelet concentration to a certain level [22].

Autogenic platelet masses stimulate collagen synthesis, induce vascular growth, reduce pain, provide hemostasis, accelerate regeneration, and reduce the risk of postoperative infectious and inflammatory complications, which has led to their use for treating soft tissue wounds and inducing reparative osteogenesis for bone defects [23–27].

Platelet concentrates are becoming more widely used in various fields of humane medicine such as orthopedics, otorhinolaryngology, gynecology, cosmetic surgery, ophthalmology, general surgery and dentistry [22, 28], as well as recently in veterinary medicine. Plasma enriched with platelets has powerful osteoinductive properties, and therefore it is combined with various osteoconductive materials [29].

Different forms of platelet mass are made by modifying the protocols of blood centrifuging. Their classification is based on two main parameters, such as fibrin architecture and cell content. Depending on this, platelet concentrates can be divided into 5 main categories [22, 33]:

- pure platelet-rich plasma (Pure Platelet-Rich Plasma (P-PRP)) or plasma rich in growth factors (Plasma Rich in Growth Factors (PRGF))

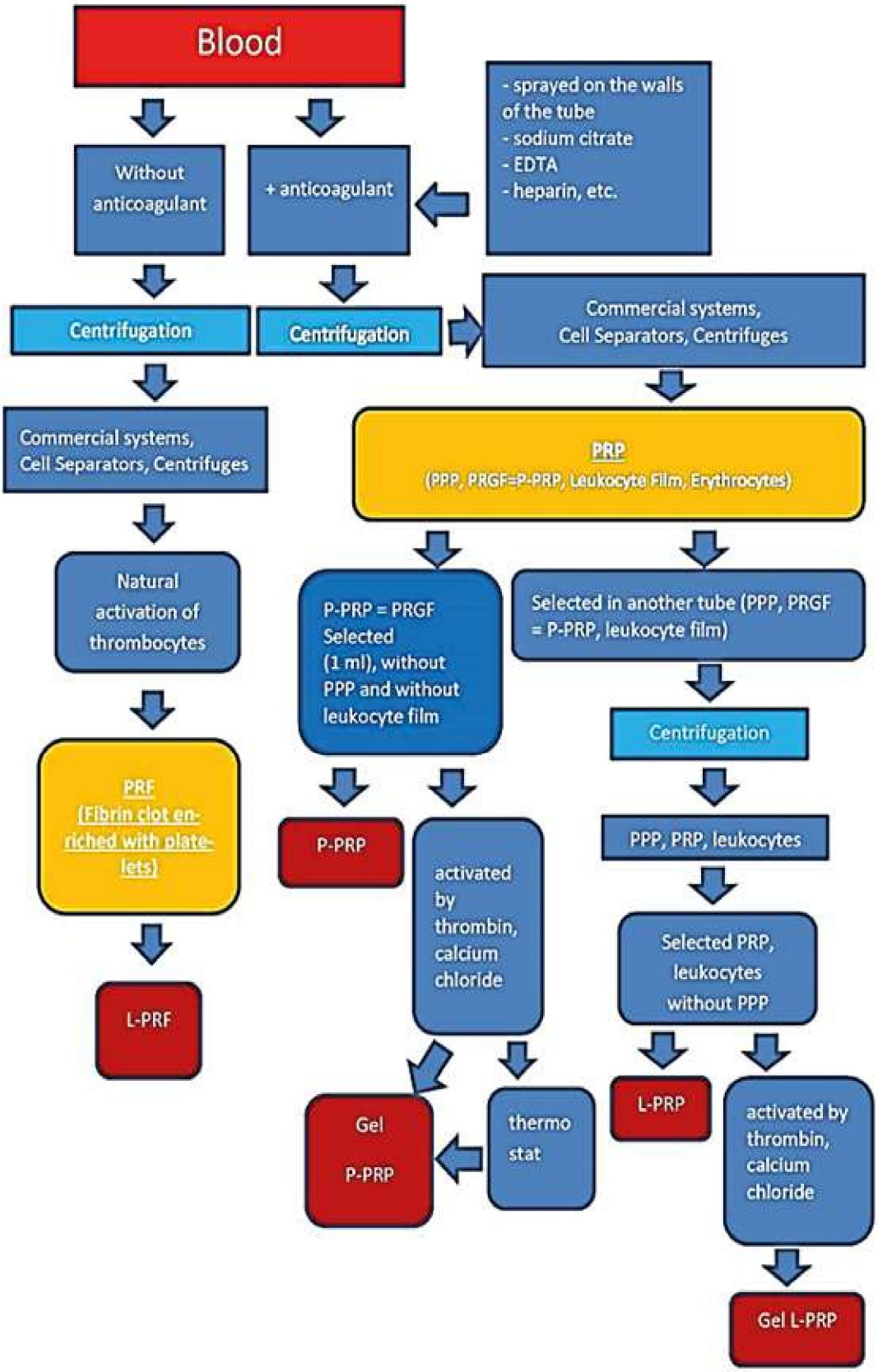

Fig. 1. **Preparation of platelet masses.**

- plasma enriched in white blood cells and platelets (Leukocyte- and Platelet-Rich Plasma (L-PRP))

- injectable platelet-rich fibrin (injectable-Platelet-Rich Fibrin (i-PRF))

- pure fibrin enriched with platelets (Pure Platelet-Rich Fibrin (P-PRF)), commercial name Fibrinet (its manufacturing technology involves the use of an anticoagulant)

- fibrin enriched with leukocytes and platelets (Platelet-Rich Fibrin (L-PRF)) [37, 38, 30].

The therapeutic efficacy of P-PRP and PRF is based on the action of a significant number of growth factors and other signaling molecules concentrated in platelet granules [39]: platelet growth factor (PDGF) [7, 40], transforming growth factor beta (TGF-b) [41], fibroblast growth factor (FGF) [37], insulin-like growth factor-1 (IGF-1) [22], insulin-like growth factor-2 (IGF-2), vascular endothelial growth factor (VEGF) [28], epidermal growth factor (EGF), interleukin 8 (IL-8), keratino-cyte growth factor (KGF) and connective tissue growth factor (CTGF) [39, 42].

Their molecules induce and regulate angiogenesis, extracellular matrix remodeling, and cellular effects: stem cell involvement, chemotaxis, proliferation, and differentiation [43]. That is, they are able to influence any stage of the regenerative process - inflammatory, proliferative and remodeling. However, the effectiveness of their influence on these biological mechanisms largely depends on the degree of release and activity of growth factors and other substances of platelets, which is determined by the technology and protocol of their concentration [28].

These forms of platelet mass are used: liquid, gel [35], clots or films (membranes) of fibrin [44, 30], topically, as an injection [45, 46, 32], or in combination with other materials [47, 29]. A variety of forms and methods of using PRP allows their use for the regeneration of various types of tissues. In this case, the type of centrifuge, its rotation speed [46, 48, 49] and rotor diameters play an important role, since platelets of different composition and properties are obtained for various parameters, which is the most controversial today [50]. The calculation of the relative centrifugal force (g) allows to determine the desired centrifugation speed for each centrifuge [51].

Platelet-rich plasma is a fraction of the volume of blood plasma after centrifugation with a platelet count within 1 million/μl of cells. Five-fold increase in the concentration of platelets is considered optimal, but at its level of more than 5 million/μl, inhibition of angiogenesis is observed [52].

The following forms of platelet-rich plasma are used: liquid — not activated and gel — activated [42, 53, 41, 49, 54]. For the concentration of platelets, one- or two-stage centrifugation is used [55, 56]. Moreover, the composition of PRP, depending on the technique, can be significantly different in the content of cells, growth factors and cytokines. It is also affected by the method of platelet activation, which affects the clinical efficacy [40, 57]. This often leads to a contradictory interpretation of its results [42].

The properties of autologous platelet-rich plasma are also affected by the rate of capture and the volume of whole blood (which determines the number of platelets in the final plasma volume), the choice of anticoagulant [46, 42] (sodium citrate, heparin, sodium EDTA and potassium EDTA (sodium and potassium ethylenediaminetetraacetic acid, which is not recommended for preparation, reduces the loss of grain size)) and pH (7.2 - 8.0) [58].

Methods for the preparation of autologous platelet-rich plasma by single-stage centri-fugation.

Blood is taken from a vein with a syringe, which is transferred to a plastic tube with an anticoagulant [37, 40]. Then the blood is centrifuged at a certain speed for a certain time (table. 1). The modes of centrifugation of blood depend on the diameter of the centrifuge rotor, as well as on the angle of its inclination [50]. There are special centrifuges for plasmolifting and test tubes with separation gel - commercial systems. After centrifugation, the erythrocyte layer (55%) separates, over which a white blood cell film (5%) and a plasma layer (40%) above it [46] are formed.In turn, the plasma is also divided into several layers - the plasma layer enriched in platelets is located immediately behind the white blood cell film, its volume is about 1/3 of the total plasma volume and the plasma layer depleted in platelets is 2/3 of the volume. After that, with the help of a syringe or a pipette, the plasma enriched in platelets is taken and transferred to another tube [29, 59, 56]. It is used either inactive (liquid), or it is activated using various substances, such as calcium gluconate, calcium chloride, cattle

Table 1– **Platelet-rich plasma techniques**

№	Name	Anticoagulant	Number of centrifugation steps	Protocol of centrifugation, (leukocyte film)	Activation	Year	Source
1	PRP	Acid dextrose of salt to lemon ACD-A acid	2	3000 rpm — (are not specified) 3 min. 4000 rpm — 3 min.	Thrombin and chloride of calcium	2018	[46]
2	PRP	Sodium citrate	2	1600 rpm — 10 min. (with leukocyte film) 2000 rpm — 10 min.	Calcium gluconate	2016	[48]
3	PRP	Sodium citrate	2	2400 rpm — 10 min. 5000 rpm — 10 min.	Calcium chloride	2018	[47]
4	PRP	Sodium citrate	2	2800 rpm — 15 min. (with leukocyte film) 2800 rpm — 15 min.	Calcium chloride	2018	[49]
5	PRP	Phosphate Citrate Dextrose	2	400 g — 15 min. (with leukocyte film) 160 g — 20 min.	not indicated	2018	[29]
6	PRGF	Sodium citrate	1	460 g — 8 min. (without leukocyte film)	Calcium chloride	2018	[55]
7	PRP	Acid dextrose of salt to lemon ACD acid	2	1800rpm — 8 min. (not indicated) 1000 rpm — 8 min.	Calcium chloride	2018	[27]
8	PRP	Sodium citrate	2	5600 rpm — 10 min. (with leukocyte film) 2400 rpm — 15 min.	Thrombin and chloride of calcium	2008	[53]
9	PRP	Sodium citrate	1	460 g — 8 min. (without leukocyte film)	Calcium chloride	2009	[60]
10	PRP	SiO	1	2800 rpm — 9 min.	not indicated	2011	[56]
11	PRP	Sodium citrate, heparin	2	200 g — 30 min. (not indicated) 450 g — 10 min.	Calcium chloride	2019	[51]
12	PRP	Sodium citrate	1	400 g — 10-12 min. (not indicated)	not indicated	2017	[59]

thrombin or other substances [48, 47, 53]. They cause the exit from the granules of platelets of various growth factors and the transformation of liquid plasma into a gel form.

Two-stage centrifugation. In the first stage, red blood cells are separated from plasma and white blood cells with platelets. During the second stage, the final separation of plasma, white blood cells and platelets occurs. As in the previous case, the blood is taken with an anticoagulant and centrifuged. The erythrocyte layer is separated, over which a white blood cell film is formed, and a plasma layer above it. The whole plasma with or without a white blood cell film (depending on what type of plasma needs to be made: pure plasma enriched in platelets or plasma enriched in white blood cells and platelets) is taken with a pipette into another tube, which is subjected to the following centrifugation (Table 1). As a result, a layer of plasma enriched in platelets is formed at the base of the tube [51]. After centrifugation, separated from the platelet-depleted plasma, the platelet mass is collected in a syringe and activated using various activators (calcium chloride, calcium gluconate, thrombin). The final product is a gel [42].

Platelet-rich fibrin.

Platelet-rich fibrin contains an autologous fibrin matrix enriched in white blood cells, platelets, and cytokines. Its tetramolecular structure acts as a matrix and is capable of biological resorption. It induces the development of the vasculature and directed cell migration [8]. If a normal clot formed from native blood without centrifugation contains 95% red blood cells, 5% platelets, less than 1% leukocytes and numerous fibrin fibers, then the platelet concentration enriched with platelets reaches 95% [61].

The advantages of PRF over other categories of platelet mass include ease of preparation,

the absence of anticoagulants and activators, uniformity and stability [25]. This autologous product does not contain any foreign substances that can adversely affect the regeneration processes and is absolutely physiological [8]. Platelet-rich fibrin is used in the form of a clot (PRF) [54, 30] and in liquid injection form (i-PRF) [45, 61, 32].

The method of preparation of fibrin enriched in platelets is presented in table. 2. It includes the selection of blood in a plastic tube without an anticoagulant and immediate centrifugation. After this, erythrocytes are concentrated in the lower part of the test tube, a fibrin clot enriched with platelets -in the middle, and serum in the upper part. It should be noted that, as in a platelet-rich plasma, a high concentration of cellular elements in a fibrin clot is located in its lower third. In the upper third, platelets can be found in very small amounts or they are absent [62, 63, 64]. The most important parameter for the success of this procedure is the minimum possible time interval between the process of blood selection and centrifugation [23, 30].

The preparation of i-PRF is based on centrifugation at low speed and the use of plastic tubes to prevent premature blood coagulation. As a result, blood is distributed into the following fractions: in the lower - erythrocytes, in the middle - red liquid, liquid fibrin enriched in platelets, in which leukocytes and platelets are more concentrated, and in the upper fraction - yellow, with fewer cellular elements [32] (table 3). After a few minutes, fibrin polymerizes and turns into a dense clot. The complexity of preparation of injectable fibrin is justified by the wider possibilities of its use in contrast to the PRF clot: in the form of an injection and in combination with other materials, forming a homogeneous composite [45, 46, 61]. However, the properties of i-PRF require detailed study [32].

The use of platelet masses.

Platelet-rich plasma is able to restore muscle tissue [26], accelerates the healing of skin wounds [67] and chronic wounds [49], reduces synovial edema, joint stiffness, which indicates its anti-inflammatory and regenerative properties [68]. The positive effect of platelet-rich plasma on tendon repair is also reported [26]. The use of platelet-rich plasma in combination with implants provides restoration of bone tissue defects [47]. Its combination withhydroxyapatite materials improved the regeneration of skull bones, as it was

Table 2 – **Platelet-rich Fibrin Preparation Techniques**

№	Name	The centrifugation protocol			Year	Source
		Rotation speed rpm	The relative centrifugal force, g	Time, minutes		
1	L-PRF	2700	not indicated	12 min	2018	[46]
2	PRF	3000	not indicated	10 min	2018	[49]
3	PRF	not specified	1843 g	10min	2018	[65]
4	PRF	not specified	400 g	10 min	2018	[54]
5	PRF	2700	735g	12 min	2019	[66]
6	PRF	2700	400 g	12 min.	2019	[32]
7	Chukrun's L-PRF	2700-3000	400 g	10-12 min	2018	[30]
8	PRF	3000	400 g	10 min	2006	[63]
9	PRF	1500	not indicated	14 min	2018	[25]

Table 3 – **Methods for the preparation of injectable platelet-rich fibrin**

№	Name	The centrifugation protocol			Year	Source
		Rotation speed rpm	The relative centrifugal force, g	Time, minutes		
1	i-PRF	700	60 g	3 min	2017	[45]
2	i-PRF	2400-2700	not indicated	2-3 min	2018	[46]
3	i-PRF	700	60 g	3 min	2019	[32]
4	i-PRF	3300	not indicated	2 min	2015	[61]

in studies on rats [29], but in some studies there were no particular advantages observed when using platelet-rich plasma [28].

Platelet-rich fibrin was used to heal wounds of the distal limbs [54]. It causes the formation of a large number of blood vessels [69], promotes the restoration of periodontal defects - a study on rats [65], and tissue repair in depulped teeth in dogs [70].

Some studies indicate results that have not demonstrated the benefits of using platelet-rich fibrin in combination with deproteinized bone material for healing bone defects [71]. At the same time, its combination with other materials gives better results in the restoration of bone defects in rabbits than the use of each of them separately [72].

Conclusions. So, platelet rich plasma and fibrin are safe, affordable, and biocompatible materials. They can be used both independently and in combination with various components. However, the effect of each of the categories of platelet mass on tissue regeneration remains debatable.

The ability of various forms of platelet masses to restore tissue due to the release of growth factors remains insufficiently studied. It is necessary to standardize the protocols for the production and classification of these autologous products. As a result of any slightest deviation from the procedure, a biologically active substance with other properties is formed.

When choosing a category of platelet masses for the restoration of a particular type of tissue, it is necessary to pay attention to the complexity of their preparation, the use of additional substances, such as anticoagulants and activators, the material of the tubes, the availability and diameter of the centrifuge, and also to consider and to optimize the possible method of administration each form of platelet mass for the regeneration of various tissues (bones, skin, muscles, etc.).

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Технології одержання тромбоцитарних мас для регенеративної медицини

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Розвиток регенеративної медицини передбачає вдосконалення існуючих та пошук нових засобів для морфофункціонального відновлення тканин, серед яких слід виділити плазму чи фібрин, збагачені тромбоцитами (PRP та PRF). Аутогенні тромбоцитарні маси стимулюють синтез колагену, індукцію росту судин, зменшують біль, забезпечують гемостаз, прискорюють регенерацію, знижують ризик післяопераційних інфекційно-запальних ускладнень, а також мають потужні остеоіндуктивні властивості.

Завдяки здатності до продукції більшості факторів росту, тромбоцити можуть впливати на всі стадії запально-регенеративного процесу, а тому їх біологічні продукти набувають суттєвого значення у вирішенні завдань регенеративної медицини. Технології виготовлення PRP та PRF грунтуються на центрифугуванні крові, в результаті чого її активні компоненти концентруються у певних ділянках центрифугату.

Відбір крові з антикоагулянтом чи без нього, а також модифікація протоколів центрифугування дозволяє одержувати різні форми тромбоцитарних мас, такі як рідина, гель або згустки. Їх класифікують, залежно від клітинного вмісту та архітектури фібрину, на декілька категорій, а саме: на чисту плазму, збагачену тромбоцитами (P-PRP) та плазму, збагачену лейкоцитами і тромбоцитами (L-PRP); ін'єкційний фібрин, збагачений тромбоцитами (i-PRF) та чистий фібрин, збагачений тромбоцитами (P-PRF), і фібрин, збагачений лейкоцитами та тромбоцитами (L-PRF).

Механізми впливу кожної із категорій тромбоцитарних мас на регенерацію тканин залишаються недостатньо вивченими. Потребують стандартизації протоколи їх отримання, із врахуванням впливу додаткових речовин, таких як активатори тромбоцитів чи згортання та антикоагулянти, а також оптимізація способів застосування кожної з форм тромбоцитарних мас.

Ключові слова: тромбоцити, PRP, PRF, центрифуги, центробіжна сила.

Технологии получения тромбоцитарных масс для регенеративной медицины

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Развитие регенеративной медицины заключается в совершенствовании существующих и поиске новых средств для морфофункционального восстановления тканей, среди которых можно выделить плазму и фибрин, обогащенные тромбоцитами (PRP и PRF). Аутогенные тромбоцитарные массы стимулируют синтез коллагена, индукцию роста сосудов, уменьшают боль, обеспечивают гемостаз, ускоряют регенерацию, снижают риск послеоперационных инфекционно-воспалительных осложнений, а также имеют мощные остеоиндуктивные свойства.

Благодаря способности продуцировать большинство факторов роста, тромбоциты могут влиять на все стадии воспалительно-регенеративного процесса, а потому их биологические продукты приобретают существенное значение в решении задач регенеративной медицины. Технологии получения PRP и PRF основываются на центрифугировании крови, в результате чего ее активные компоненты концентрируются в определенных участках центрифугата.

Отбор крови с антикоагулянтом или без него, а также модификация протоколов центрифугирования позволяет получать различные формы тромбоцитарных масс, такие как жидкость, гель или сгустки. Их классифицируют, в зависимости от клеточного содержания и архитектуры фибрина, на несколько категорий, а именно: чистую плазму, обогащенную тромбоцитами (P-PRP), плазму, обогащенную лейкоцитами и тромбоцитами (L-PRP); инъекционный фибрин, обогащенный тромбоцитами (и-PRF) и чистый фибрин, обогащенный тромбоцитами (P-PRF), а также фибрин, обогащенный лейкоцитами и тромбоцитами (L-PRF).

Механизмы влияния каждой из категорий тромбоцитарных масс на регенерацию тканей остается недостаточно изученым. Необходимы стандартизация протоколов их получения с учетом влияния дополнительных веществ, таких как активаторы тромбоцитов или сворачивания крови и антикоагулянты, а также оптимизация способов применения каждой из форм тромбоцитарных масс.

Ключевые слова: тромбоциты, PRP, PRF, центрифуги, центробежная сила.

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