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CLINICAL PROTOCOL FOR THE PREPARATION AND ELECTRON MICROSCOPIC ANALYSIS OF THE OBTAINED PRODUCTS OF AUTOLOGOUS MESOCONCENTRATE – PLASMA RICH IN GROWTH FACTORS (PRGF)

Anatolii P. Oshurko[1], Ihor Yu. Oliinyk[1], Tetiana M. Kerimova[2], Elina S. Pompii[2]

1 – Bukovinian State Medical University, Chernivtsi, Ukraine
2 – SE «Lugansk State Medical University», Rivne, Ukraine.

Summary

Introduction. Ensuring control over regenerative processes by using the patient's blood is a unique concept of an autogenous transplant product, a universal and safe method of application that contains growth factors and cytokines stored in the fibrin matrix and provides stimulating properties of tissue regeneration through the processes of angiogenesis, proliferation, cell migration, and extracellular matrix synthesis.
The aim. To substantiate the effectiveness of the protocol for collecting, preparing, and forming autocellular mesoconcentrate products in the analysis of the results of their electron microscopic examination.
Materials and methods. Based on our own clinical experience of using the Endoret-PRGF technique (Human Technology, BTI, Spain) to collect, prepare, and form autocellular transplant products rationally, we used the method of morphological study of objects using a stream of electrons that passed through thin films under high voltage, allowing us to study the structure of these objects at the macromolecular and subcellular levels – transmission electron microscopy**,** which contributed to the study of the density (number of occurrences in 10 μm^2), diameter (Ø) of the formed fibrin fibers in the PRGF – F1 and F2 fractions of mesoconcentrate products, namely, isolating membranes (M) and obturating blocks (B). An equally important step in PRGF technology is to follow the step-by-step instructions for preparing and using autocellular transplants.
Results. Protocol for the collection, preparation, and formation of autocellular transplants using the KMU15 kit (Human Technology, BTI) for the application of the Endoret – PRGF technique, platelet-rich plasma, as a unique and first scientifically proven technique recognized worldwide and patented by the Institute of Human Biotechnology, Spain. The results of the clinical application of mesoconcentrate products, namely isolating membranes widely used in targeted tissue regeneration and obturating blocks, confirm their biological effectiveness. The results of the *median* (Me) *and interquartile range* (IQR) during the statistical analysis were obtained, where **F1-M** = 0.196 (0.176; 0.286) compared to **F1-B** = 0.344 (0.325; 0.394); **F2-M** = 0.180 (0.168; 0.214) – **F2-B** = 0.254 (0.202; 0.338), which gives us the right to assert the reliability of the data set and confirms the quantitative characterization of interfraction differences, and, accordingly, the quality in the sequence of protocol stages.
Conclusions. The protocol for the efficiency of autocellular graft collection and formation is synchronized with the analysis of electron microscopic images characterizing the **density and** diameter of fibrin fibers, which differ in the fractional products of the mesoconcentrate and are higher in the obturating blocks than in the isolating membranes, indicating its completeness and compliance.

Key words: **atrophy, bone tissue, PRGF (Plasma Rich in Growth Factors), autocellular transplants**

INTRODUCTION

Today, clinicians have a wide range of methods and techniques of autocellular transplantation available to help them achieve their goals and solve specific problems. However, due to the variability and volume of their clinical protocols and technical and economic support problems, the latter deprives the doctor of the opportunity to use them properly, or their separate fragmentary use does not yield the expected results.

The growing interest of researchers in the priority use of mesoconcentrate is demonstrated by a significant number of scientific publications on this issue [3, 4, 6]. It is important to note that there are clinical observations and a growing number of experimental studies [2, 7, 8].

According to the undisputed data of the world medical statistics, the results applying seemingly similar PRF (Platelet Rich Fibrin) and PRGF (Plasma Rich in Growth Factors) are in the first place in such studies. That is, obtaining platelet-derived growth factors, which are dimeric glycoproteins, from the patient's venous blood by centrifugation, which separates plasma with platelets from other blood cells, which may contain both platelets and leukocytes, which to some extent differentiates the methods from each other.

The common mechanisms for these methods are the synthesis and formation of platelet-derived growth factors in bone marrow cells, i.e., megakaryocytes, as platelet precursors, with their accumulation in platelet alpha granules (blood plates). When interacting with thrombin, blood platelets are activated, and their contents are released into the blood plasma. These glycoproteins exert their primary mitogenic effect on cells of mesenchymal origin, including fibroblasts and glial cells, stimulating the onset of their division. However, a significant difference is characterized by the concentration of glycoproteins in the fractional distribution of PRGF (F1, F2) obtained by these plasma methods, a critical feature in their selection and practical application.

THE AIM

To conduct an electron microscopic analysis of the results of the obtained cell autotransplantation products and to substantiate the effectiveness of the protocol of collection, preparation, and formation in morphological terms.

MATERIALS AND METHODS

The work is based on the analysis of a freely available method of obtaining platelet-rich plasma, based on our own clinical experience, namely, the Endoret-PRGF technique (Human Technology, BTI, Spain), for rational collection, preparation, and formation of autocellular transplant products for widespread clinical implementation. The details of our protocol for the collection and formation of autocellular transplants, which are presented below, are based on compliance with moral and ethical standards and have no signs of a conflict of interest between the authors and the patent holder, which is confirmed by the signing of the international agreement on scientific cooperation No. 12-05/10 of 17.11.2021, on writing a doctoral dissertation on the topic: «Substantiation of rehabilitation of patients with bone atrophy complicated by topographic and anatomical features of the mandibular canal».

To study the density (number of occurrences in 10 μm^2) and diameter (Ø) of the formed fibrin fibers in the PRGF fractions F1 and F2 of the mesoconcentrate products, namely, isolating membranes (M) and obturating blocks (B), we used the method of morphological study of objects using a stream of electrons, which, under the influence of high voltage, passed through thin films, allowing us to study the structure of these objects at the macromolecular and subcellular levels – transmission electron microscopy.

Preparation for microscopic examination was carried out in the following sequence. Samples of the obtained membranes and blocks were fixed in a 1.5 % solution of osmium tetroxide in 0.2 M cacodylate buffer (pH 7.2) in the cold for 2 hours. The fixed cultures were washed 3 times with buffer solution. The resulting fixed materials were dehydrated in increasing concentrations of ethyl alcohol (25 %, 50 %, 70 %, 90 %, and 96 %), each time for 10 min, and released with an acetone solution twice for 30 min. Epoxy resins (Fluka) were mixed in the ratio of Epon 812-4.5 ml, DDSA – 2.2 ml, and MNA – 2.2 ml. The material under study was enriched with four mixtures of epoxy resin and acetone in the ratios of 1:3, 1:2, 1:1, and 2:1. The exposure time of the final enrichment was 14 hours, after which it was transferred to a polypropylene capsule made of a freshly prepared epoxy resin mixture containing a catalyst (5 drops of DMP – 30 per 10 ml of mixture) and polymerized in a thermostat sequentially at 37 °C (12 hours) and then at 60 °C (48 hours). Sections were prepared on an LKB Ultrotome III ultramicrotome using a glass knife and transferred to nickel grids. Sections were contrasted sequentially in a 2 % alcohol solution of uranyl acetate and lead citrate.

Microscopic selective evaluation was carried out using an electron transmission microscope PEM-100-01 with a voltage of 75 kV at magnifications from ×1000 to ×10000.

Morphometric studies of fibrin filaments were performed using distance measurements on electron micrographs of the tissue ultrastructure obtained by transmission electron microscopy. A 1 μm scale was applied to each photograph, which served as a standard for recording distances on the micrographs.

The ultrastructure image on the electron micrographs was viewed at a magnification of x3000 and a square frame with a side of 3.16 μm and an area of 10 μm^2. The frame was fixed in the area of the micrograph with the «average» concentration of fibrin filaments for a given tissue sample. Within the frame, cross-sections were drawn for 10 different filaments. The density of fibrin filaments was determined by quantifying them in a selected square. The dimensions of the cross-sectional sections of the filaments were recorded in a table for further statistical analysis.

Statistical data analysis was used to determine the mean morphometric values *(M)* for each selected fraction, their products, and the standard error of the mean values *(±m)*. Comparison of the obtained values of interfraction products of the mesoconcentrate was carried out using nonparametric methods, using the Mann-Whitney U test, since the sample differed from the normal distribution.

This paper complies with the provisions of the Law of Ukraine No. 2297-VI «On Personal Data Protection» dated June 1, 2010, as amended by the Laws of Ukraine No. 4452-VI dated February 23, 2012, and No. 5491-VI dated November 20, 2012. No. 5491-VI, which regulate legal relations related to the protection and processing of personal data and is aimed at protecting the fundamental rights and freedoms of man and citizen and compliance with the basic provisions of the GDPR, the World Medical Association's Declaration of Helsinki on the Ethical Principles for Scientific Medical Research Involving Human Subjects (1964-2013). The planned direction of these studies was reviewed by the Commission on Biomedical Ethics of Bukovinian State Medical University (Chernivtsi, Ukraine) and approved by Protocol No. 2 of 21.10.2021.

RESULTS

We have achieved the most predictable clinical results when applying the Protocol for the collection, preparation, and formation of autocellular grafts using the KMU15 kit (Human Technology, BTI) for the application of the Endoret-PRGF technique (Figure 1), platelet-rich plasma, as a unique and first scientifically proven technique recognized worldwide and patented (patent No. 1066838) by the Institute of Human Biotechnology, Spain.

1. Blood collection. Blood is collected with the Vacutainer system (Figure 1, I) into nine-millimeter extraction tubes (with blue caps), which are available in each set of four pieces containing calcium citrate (Figure 1, II). If you use an odd number of tubes, add one tube filled with water to ensure the rotor is adequately balanced during centrifugation.

2. Centrifugation. Place the tubes with the «biological fluid-blood» into the centrifuge cups, balancing the rotor correctly (Figure 1, III). To perform this step, use a PRGF System V centrifuge at the programmed speed parameters for 9 ml tubes (T9).

After centrifugation, the resulting ratio of blood cells to plasma may vary, depending on the individual physiological characteristics of the patient's blood. If the resulting plasma is whitish or red (Figure 2), it is strongly recommended not to use it.

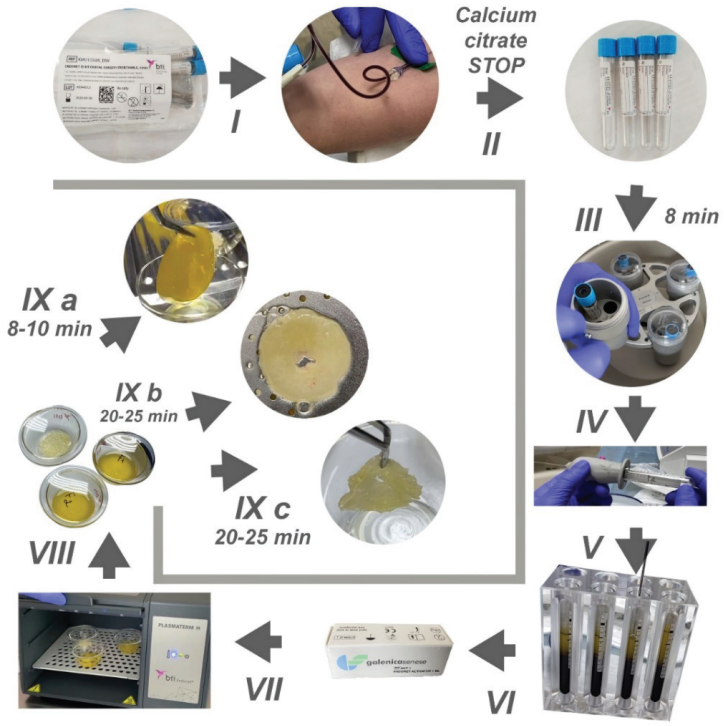


Fig. 1. Protocol for collecting, preparing, and forming autocellular grafts using the Endoret-PRGF, BTI technique

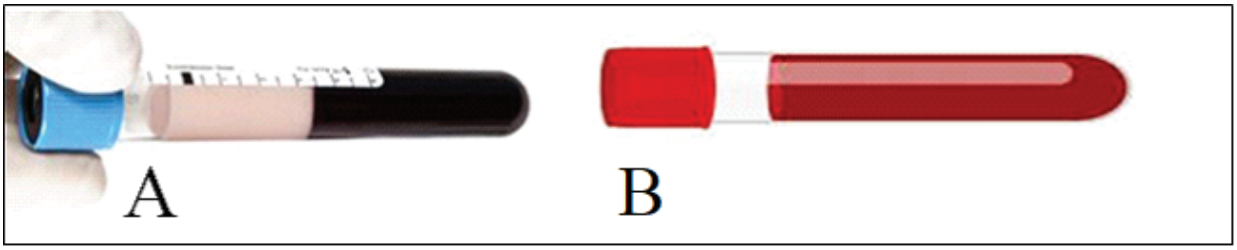


Fig. 2. A – hypercholesterolemia; B – erythrocyte hemolysis

3. Division into fractions. Label the two fraction collection tubes (with white caps) as F1 and F2 to prevent possible errors. After centrifugation, mark the scale of each of the four tubes with blue lids: 1 – the first mark is the dividing line between the plasma fraction and the leukocyte film (0.5 ml above the upper limit of the red blood cell level); 2 – the second mark is the line dividing the two fractions into F1 and F2. The fractionation process uses a «plasma transfer device – PTD2» (Figure 1, IV). Pull out the protective black wing outside the tube before inserting the first fraction (F1) collection tube into the PTD2. The pink button on the PTD2 device should be pressed only when the end of the lead is immersed in plasma to avoid filling the fraction tube with air and losing its vacuum, gradually deepening it at the rate of fraction aspiration. The volume of the second fraction (F2) will always be 2 ml of platelet-rich plasma in each tube, while the volume of the first fraction (F1) will vary depending on the patient's blood properties. The first plasma fraction is aspirated from each mesoconcentrate extraction tube to the indicated mark (Figure 1, V). After connecting the fractional labeled F1 tube with a white cap to the PTD2 plasma transfer device, aspirate the upper fraction of the F1 plasma from each centrifuged biomaterial tube to the previously marked line separating the two fractions. Also, by connecting the labeled F2 tube to the PTD2 device, we complete the aspiration, 2 ml from each extraction tube of the second fraction of F2 plasma enriched with platelets.

4. Plasma activation is performed using a PRGF activator (Figure 1, VI). Each 1 ml of plasma is activated by adding 2 units of activator (Table 1), for which we use the graduated syringe provided in each kit.

Table 1

Ratio of plasma (ml) to PRGF activator (units of the activation syringe scale, un. act. sc.)

Activation	Plasma, ml	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0
	Activator, un. act. sc.	1.0	2.0	4.0	6.0	8.0	10.0	12.0	14.0	16.0	18.0

Activated PRGF in the liquid state (second fraction, F2) is possible within 8 minutes after activation.

The formation of a clot from the second fraction (Figure 1, IX-a) will occur 8-10 minutes after the addition of the activator, while the fibrin membrane (Figure 1, IX-b) from the first fraction (F1) of PRGF will be obtained in 20-25 minutes. To prepare a bone graft (Figure 1, IX-c), it is necessary to mix newly activated liquid PRGF (fraction F2) with bone-plastic or autogenous bone biomaterial in a ratio of 1:2 (0.5 g of biomaterial per 1 ml of fraction F2).

The Institute of Human Technology, BTI, Spain, has issued warnings regarding using the proper amount of activator in case of deviation of the ratio, inhibition, or changes in the cascade of plasma coagulation reactions into a clot. It is also recommended to use special BTI process glass containers for clot formation, fibrin membrane, and/or graft preparation. This is justified by the fact that when glass containers with activated plasma are placed in the Plasmaterm H furnace (Figure 1, VII), optimal temperature conditions (37° C) are created for the reactions of formation of all «products» derived from plasma, which fully correspond in speed to similar physiological processes of the human body.

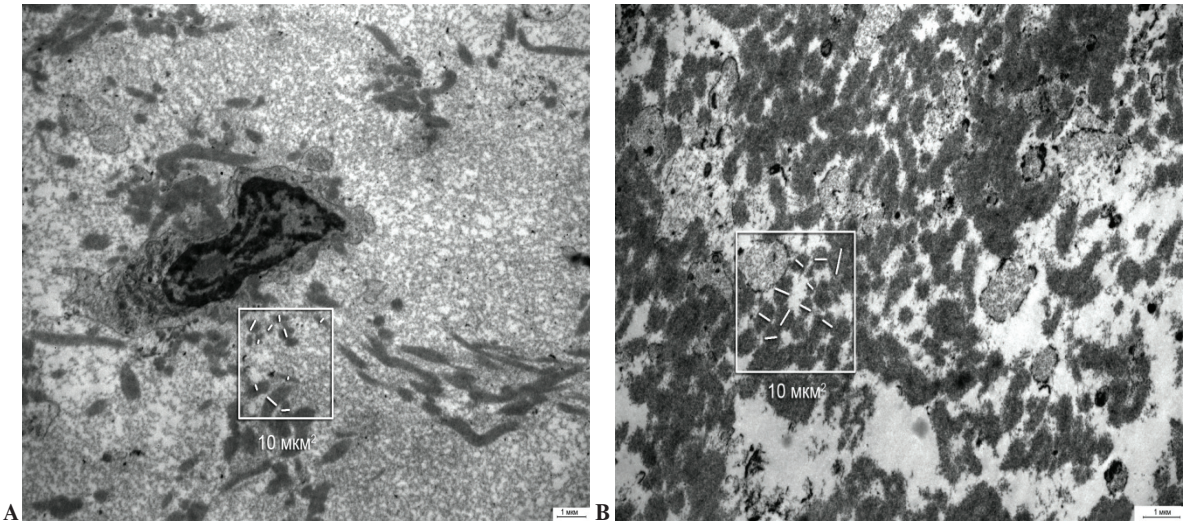
An equally important step in PRGF technology is to follow the step-by-step instructions for preparing and using autocellular transplants. After blood is collected from the recipient donor, it must be centrifuged within 1 hour. The resulting mesoconcentrate is immediately divided into fractions. Fractionated plasma can be activated within four hours of separation.

To achieve proper results of autocellular transplantation, it is recommended to follow the rules of asepsis and antisepsis, ensuring sterility and disposal of biological waste in accordance with the country's current legislation.

The proven effectiveness of clinical use of mesoconcentrate products, namely isolating membranes, which are widely used in targeted tissue regeneration – closing bone augmentation, burn zones after surgery, neurovascular structures in areas of exposure, etc, and obturating blocks – for closing oroantral connections, compensation of lost tissues, as intended, confirms its biological effectiveness, with the understanding of transmission electron microscopy, even when visualized on images (Figure 3, Figure 4).

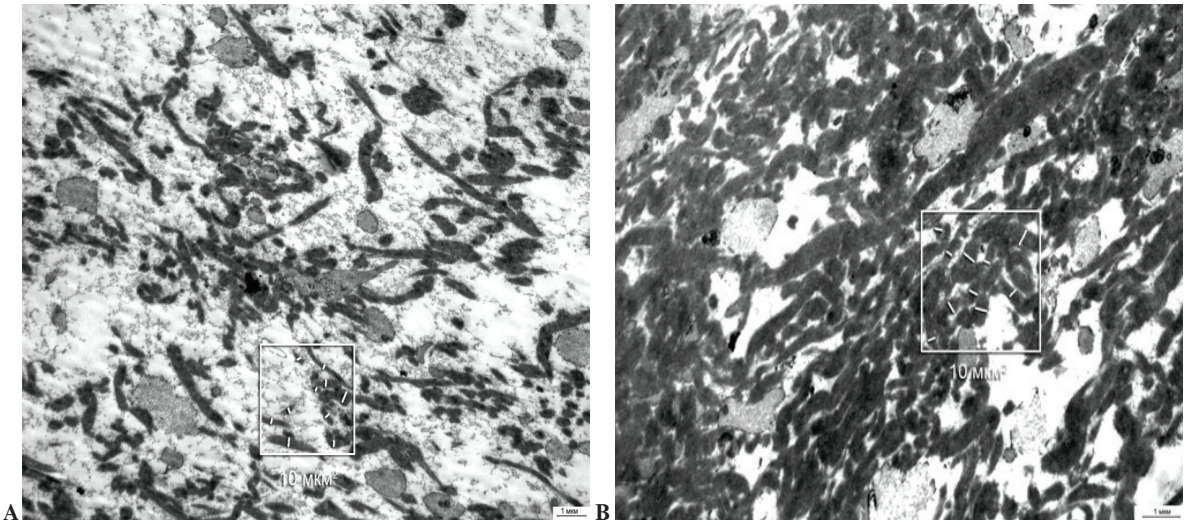
The density and diameter of fibrin filaments, which are an autogenous and fundamental mesenchymal framework for further tissue construction, in particular, ensuring the sustainability of regenerative processes of damaged bone tissue by multiethiological factors, is significantly differentiated both in its clinical purpose and in the analysis of electron microscopy between PRGF F1-M and PRGF F1-B fractions, as well as PRGF F2-M and PRGF F2-B, the values of which are presented in Table 2.

For statistical comprehensiveness, we conducted a parallel analysis to determine the median and interquartile range, which amounted to: Me (IQR), where **F1-M** = 0.196 (0.176; 0.286) – **F1-B =** 0.344 (0.325; 0.394); **F2-M** = 0.180 (0.168; 0.214) – **F2-B** = 0.254 (0.202; 0.338), which gives the right to assert the reliability of the data set and confirms the quantitative characterization of interfraction differences.



A B

Fig. 3. Transmission electron micrograph with determination of the cross-sectional diameter of fibrin fibers and their density. Magnification x3000: A) PRGF F1-M; B) PRGF F1-B



A B

Fig. 4. Transmission electron micrograph with determination of the cross-sectional diameter of fibrin fibers and their density. Magnification X3000: a) PRGF F2 – M, C) PRGF F2 – B

Table 2

Results of morphometric study of diameter (Ø) and density in 10 μm² of formed fibrin filaments in the fractional distribution of PRGF, mesoconcentrate membrane products (M), and obturating blocks (O)

Value	PRGF (F1-M), (Ø)	PRGF (F1-B), (Ø)	PRGF (F2-M), (Ø)	PRGF (F2-B), (Ø)
M, ± m	0,232±0,033	0,362±0,032	0,232±0,022	0,269±0,024
p	0.019		0.024	
N=10 μm²	16.0	18.0	29	29

DISCUSSION

Accurate adherence to the PRGF (Plasma Rich in Growth Factors) protocol presented by the authors [12] provides an excellent clinical prognosis in even complex surgical interventions, in particular, in combined bone transplantation [1] and in the treatment of acquired bone atrophy as a consequence of early loss of the masticatory group of teeth [10], which leads to pronounced topographic variability of important morphological structures [9].

We are aware of the PRF method, which remains in the space of scientific discussions and practical application, presented in 2001 by Dr. Joseph Choukroun (France) – a platelet enrichment method clinicians widely used for targeted regeneration of soft and hard tissues.

In 2014, the author and his team, in collaboration with the FORM laboratory (Frankfurt) and the Clarion research laboratory (USA), presented the following discovery at an international symposium (Paris) – the i-PRF method (injectable platelet-rich fibrin), the results of which were tested and presented in the author's research. Although it impresses with its simplicity and ergonomics described by many researchers [11], it is characterized by differences in the clinical protocol, particularly the formation of mesoconcentrate end products. Its multiclinical use makes it impossible to adhere to the proposed PRF protocol's accuracy, leading to a significant error in the final result.

The authors of [7], in a systematic review of the clinical literature based on the Stedman Research Institute of Phillipton, Colorado, and the Cleveland Clinic Foundation, Ohio, call for standardization of protocols for platelet-rich plasma in its widespread use. Although their work is highly cited, in my opinion, the researchers have not been able to systematize everything into a single whole to meet the requirements of technical support and its universality in accessibility and clinical application.

The principle of biological expediency unites well-known methods in clinical application. After all, the donor and the recipient are one macroorganism, that is, the patient himself, who is permanently «available» and eliminates significant clinical risks regarding contraindications to transplantation, and additional paraclinical research methods.

The principle of physiological capacity has the same characteristic. Rapid autocompensation in the restoration of circulating blood volume leaves no signs of iatrogenic effects on the body, which emphasizes its minimally invasive nature, but a significant concentration of leukocyte content in the PRF mesoconcentrate, as opposed to PRGF, negatively affects regenerative processes, as their focus is on localized action.

CONCLUSIONS

1. Density of fibrin filaments in 10 μm² in fractions F1-M = 16 pcs. vs. F2-M = 29 pcs., F1-B = 18 pcs. vs. F2-B = 29 pcs., which confirms the differentiation in their fractional concentration and productivity in application.

2. The diameter of fibrin fibers differs in the fractional products of the mesoconcentrate and is higher in the obturating blocks than in the isolating membranes, indicating compliance with the protocol of their preparation and preservation of normal physiological processes in the body tissues targeted for controlled autologous transplantation.

Prospects for further research include studying the qualitative characteristics of tissues, including bone tissue, that have been subjected to directed regeneration using autocellular transplants, in particular, mesoconcentrate products.

FUNDING AND CONFLICT OF INTEREST

The authors declare that there is no conflict of interest and assure that this work was conducted as part of the initiative research work of the Department of Histology, Cytology and Embryology of Bukovinian State Medical University «Structural and functional features of tissues and organs in ontogeny, patterns of variant, constitutional, sex-age and comparative human morphology», state registration number 0121U11012.

COMPLIANCE WITH ETHICAL REQUIREMENTS

This paper complies with the provisions of the Law of Ukraine No. 2297-VI «On Personal Data Protection» dated June 1, 2010, as amended by the Laws of Ukraine No. 4452-VI dated February 23, 2012, and No. 5491-VI dated November 20, 2012. No. 5491-VI, which regulate legal relations related to the protection and processing of personal data and is aimed at protecting the fundamental rights and freedoms of man and citizen and compliance with the basic provisions of the GDPR, the World Medical Association's Declaration of Helsinki on the Ethical Principles for Scientific Medical Research Involving Human Subjects (1964-2013). The planned direction of these studies was reviewed by the Commission on Biomedical Ethics of Bukovinian State Medical University (Chernivtsi, Ukraine) and approved by Protocol No. 2 of 21.10.2021.

AUTHOR'S CONTRIBUTION TO THE ARTICLE

Anatolii P. Oshurko:A, B, C, D, E

Ihor Yu. Oliinyk: A, B, E, F

Tetiana M. Kerimova: B, C, Д, E

Elina S. Pompii: B, C, Д, E

A – Work concept and design,

B – Data collection and analysis,

C – Responsibility for statistical analysis,

D – Writing the article,

E – Critical review,

F – Final approval of the article

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Резюме

КЛІНІЧНИЙ ПРОТОКОЛ ПІДГОТОВКИ ТА ЕЛЕКТРОННО-МІКРОСКОПІЧНИЙ АНАЛІЗ ОТРИМАНИХ ПРОДУКТІВ АУТОЛОГІЧНОГО МЕЗОКОНЦЕНТРАТУ – PLASMA RICH IN GROWTH FACTORS (PRGF)

Анатолій П. Ошурко[1], Ігор Ю. Олійник[1], Тетяна М. Керімова[2], Еліна С. Помпій[2]

1 – Буковинський державний медичний університет, м. Чернівці, Україна
2 – ДЗ «Луганський державний медичний університет», м. Рівне, Україна

Вступ. Забезпечення контролю над регенераторними процесами шляхом використання власної крові пацієнта є унікальною концепцією продукту аутогенної трансплантації, універсальної і безпечної методики застосування, яка містить фактори росту й цитокіни, що зберігаються в матриці фібрину та забезпечують стимулюючі властивості регенерації тканин за допомогою процесів ангіогенезу, проліферації, клітинної міграції та синтезу позаклітинного матриксу.
Мета. Обґрунтувати ефективність протоколу забору, підготовки та формування продуктів аутоклітинного мезоконцентрату у проведеному аналізі результатів їх електронно-мікроскопічного дослідження.
Матеріали та методи. За вибором власного клінічного досвіду застосування техніки Endoret – PRGF (Human Technology, BTI, Іспанія), з метою раціонального забору, підготовки та формування продуктів аутоклітинних трансплантатів, нами застосовано метод морфологічного дослідження об'єктів за допомогою потоку електронів, які під дією високої напруги проходили крізь тонкі плівки, дозволяючи вивчати структуру даних об'єктів на макромолекулярному і субклітинному рівнях – трансмісійної електронної мікроскопії, що сприяло проведенню дослідження щільності (кількість залягання в 10 мкм^2), діаметру (Ø) сформованих фібринових волокон у фракціях PRGF – F1 та F2 продуктів мезоконцентрату, а саме, ізолюючих мембран (М) та обтуруючих блоків (В). Не менш важливим етапом у технології PRGF є дотримання покрокової інструкції щодо приготування та застосування аутоклітинних трансплантатів.
Результати. Протокол забору, підготовки та формування аутоклітинних трансплантатів із використанням набору KMU15 (Human Technology, BTI) для застосування техніки Endoret – PRGF, плазми збагаченої тромбоцитами, як унікальної і першої науково обґрунтованої методики, визнаної у всьому світі та запатентованої Інститутом Біотехнології людини, Іспанія. Результати клінічного застосування продуктів мезоконцентрату, а саме ізолюючих мембран, що широко застосовуються у направленій тканинній регенерації та обтуруючих блоків, підтверджують свою біологічну ефективності. Отримані результати *медіани* (Me) *та інтерквартильного розмаху* (IQR) під час статистичного аналізу, де **F1-M** = 0,196 (0,176; 0,286) у порівнянні з **F1-B =** 0,344 (0,325; 0,394); **F2-M** = 0,180 (0,168; 0,214) – **F2-B** = 0,254 (0,202; 0,338), що дає право стверджувати про достовірність набору даних та підтверджує кількісну характеристику міжфракційних відмінностей, а, відповідно, і якість у послідовності протокольних етапів.
Висновки. Протокол ефективності забору та формування аутоклітинних трансплантатів є співсинхронним із аналізом електронних мікроскопічних зображень, які характеризують щільність, діаметр фібринових волокон, що відрізняються у фракційних продуктах мезоконцентрату і є вищим у обтуруючих блоках, ніж в ізолюючих мембранах, що вказує на його повноту та дотримання.

Ключові слова: **атрофія, кісткова тканина, PRGF (Plasma Rich In Growth Factors), аутоклітинні трансплантати**