UDC 615.382: 612.111.7

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Standardization of platelet aggregation tests to evaluate condition of hemostasis

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The aim of the study was to develop the method of quantitative evaluation of the platelets state in patient based on their aggregation and implement it to the medical practice. The method is based on analysis of the following parameters: strength of the created clot, its disaggregation and calculation of the quantitative index, i.e. platelet functional activity index (PFAI). Than authors evaluated the aggregation features of platelets by PFAI in combination with their quantitative content in peripheral blood followed by identification of normal, hyperactive and hypoactive platelet unit of hemostasis. Eventually, the new algorithm for plasma rich growth factors application was developed and implemented based on features mentioned above. It can be used for treatment of patients at the departments for maxillofacial surgery and dentistry, providing good or satisfactory results in the absolute majority of patients. Evaluation of platelet unit of hemostasis functional state based on PFAI provides the opportunity to standardize the platelet aggregation method. The suggested method is based on the biological principle of maintaining the most important constants of homeostasis and can be recommended for the development of more effective therapeutic approaches in clinical practice using the regenerative potential of platelets.

Key words: platelet functional activity; disaggregation; platelet; plasma; rich in growth factors.

INTRODUCTION

Understanding of hemostasis mechanism in the damaged tissues as a cascade of biochemical interactions of plasma factors under the theory of "internal" and "external" path has been widely recognized in academic life and laboratory practices. The same occurred in the research of hemostasis separate stages i.e. vascular-platelet turning to coagulation (in plasma), where sequential stages of platelet activation are clearly indicated, followed by formation and accumulation of insoluble fibrin, and finally by removal of excessive fibrin by fibrinolysis [1]. Although many authors emphasize that this division in stages and processes is largely conditional, and the process of maintaining homeostatic balance and liquid blood state is much more complex and harmonized [1-3].

Supporters of the "cellular" theory of homeostasis note that the concept of coagulation cascade is erroneous and does not reflect the model of homeostatic process in vivo [4]. To ensure hemostasis, a local concentration of the platelets and fibrin in the site of vessel damage, as well as activated procoagulant components, which are necessary to ensure thrombus formation are not controlled throughout the entire vascular system. And since, procoagulant reactions can be realized only on certain cell surfaces, the "cellular" model offers sequentially overlapping stages: initiation, amplification, propagation resulting in the formation of large amounts of thrombin, the so-called "thrombin explosion" [5]. Researchers, who understand hemostasis in such a way, emphasize on the central role of the cell surface of the platelets and the importance of specific receptors [5-7].

Platelet aggregation is actively studied in laboratory practices, being the gold standard for the test of platelet function as well as for the diagnosis for hemostasis disorders in patients [8]. Since dif-

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ferent types and doses of aggregation activators have been used in various studies, clinical results can often not be fully comparable [8,9].

Nowadays, alternative methods have been developed for measuring platelet aggregation in platelet-rich plasma or whole blood e.g. impedance aggregation, aggregation plate with 96 cells, flow cytometry [10].Some of them allow faster and more convenient test of specific platelet reactions in response to the addition of inducers. However, these methods are not widely used and, unlike aggregation, they do not provide important diagnostic information that can identify platelet functional defect, the emergence of secondary wave, or characterize platelet disaggregation.

But despite the popularity and wide application, the method of aggregation is not standardized [9]. There are several methodological differences in main principles of the evaluation of the platelets functional activity [8,10]. This state of things forces the international professional communities to develop consensus recommendations on the standardization of the methodology, to create Guidelines [11,12], providing clinicians with clear guide for testing platelet function in patients with suspected hemostasis systemic impairments [11-13].

The need to adapt aggregation data for clinicians encourages researchers to modify the technique, add input data, calculate coefficients, etc. [14,15]. Therefore, some studies are aimed at collecting scientific data on how to choose the most appropriate procedures for the implementation of aggregation tests. The need for methodological standardization also remains relevant.

Purpose: develop a method for quantitative evaluation of the platelet hemostasis state in patients on the basis of their aggregation and introduce it to clinical practice.

METHODS

The study included 30 patients treated at Kyiv Regional Center for Maxillofacial Surgery and Dentistry for post-traumatic and postoperative upper and lower jaw defects aged between 17 and 70, with an average age of 36.2 ± 13.4 years old. Men accounted for 43.3% of the total number of those surveyed. The individual card of each patient included information about age, sex, presence of concomitant somatic diseases and bad habits, size of existing defect and its etiology, use of anticoagulants and non-steroidal anti-inflammatory drugs, etc.

The patients underwent the complex clinical and laboratory test, which included clinical blood test and aggregation test prior to surgical intervention.

All laboratory and diagnostic tests were performed at clinical diagnostics laboratory (headed by I.N. Ryzhko) at Research Institute for Experimental and Clinical Medicine in Bohomolets National Medical University (Kyiv) based on standard methods.

Hemogram was obtained by hematology analyzer MicroCC, China. The test of platelet aggregation was carried out by performing the induced aggregation of platelets in plasma on analyzer PM-2110 Solar, Belarus. ADP solutions of Technology-Standard Inc. (Russia) at a final concentration of 0.5 μm and Sigma adrenaline hydrochloride solution at concentration of 5.0 μm were used as inducers of aggregation.

The blood for research was collected from patients by vacuum method into VACUETTE plastic tubes manufactured by Grenier-bio-one, Austria containing the anticoagulant (5.5% aqueous solution of sodium citrate 3.8%), with ratio of blood to anticoagulant 9:1.

Plasma with various platelet concentrations was obtained for each patient individually. To obtain platelet enriched plasma the tubes were centrifuged for 5 minutes at 200 G mode, while plasma was taken out followed by centrifuging the tubes again for 10 min at 2000 G mode to receive platelet poor plasma. Eventually the resultant poor platelet plasma was applied for device calibration, by its dilution to required level for platelet concentration measuring. Recording of induced platelet aggregation was performed according to the instructions to the aggregometer.

Thus, a magnetic stirrer was added into plastic cuvette containing 270 μl of platelet enriched plasma and heated for 5 minutes at 37.0 C° in the section for samples preparation. Having transferred the cell with plasma to the measuring window of a calibrated aggregometer, an inductor was added, and recording was initiated by pressing START button. The changes in the aggregation curve were observed. When the recording was over, the aggregation test was processed by SOLAR software.

Statistic package MedCalc ver. 16.8 (2016) was used in order to define the correlation connection. The Pearson correlation coefficient (r) was calculated, with a P-value of 95% confidence interval for the correlation coefficient.

REZULTS AND DISCUSSION

Standard parameters of aggregation curve were automatically calculated after registration of the aggregation research assisted by SOLAR software: the degree of aggregation (%), i.e. the maximum level of relative light transmission of plasma followed by aggregation inducer administration; aggregation time (AT, min), i.e. time corresponding to the maximum degree of aggregation after pressing the "START" button; aggregation rate (%/min), i.e. the change in the relative light transmission of plasma after introduction of aggregation inducer, measured within 30 s (along the abscissa) from the selected reference point.

However, it is recommended to analyze not only the features of platelet aggregation, but also the strength parameters of created clot considering its disaggregation to evaluate the state of platelet hemostasis and also to compare the aggregation properties of platelets with their quantitative content in peripheral blood, i.e. PLT index obtained in hemogram report. Parameters received by SOLAR software are not enough for this. Therefore, an original method for evaluation of platelets functional properties was developed, considering the aggregation and disaggregation properties of platelets.

The method is realized in the following way: the degree of disaggregation (%) as the difference between the maximum on aggregation curve and the lowest point on curve after this maximum is measured additionally. If the curve has several maxima, the degree of disaggregation is counted after each maximum and is summed.

After that, disaggregation time (min), i.e. time for the very disaggregation process is measured. The average value of aggregation degree (AD) and aggregation rate (AR) in the pool of healthy donors (not less than 20) is measured to identify the ratio of AD and AR of control (donors) showing AD/ADd and AR/ARd accordingly. Aggregation efficiency (AF) is identified as Aggregation degree (AD) divided by Aggregation time (AT), disaggregation efficiency (DE) as Disaggregation degree (DD) divided by Disaggregation time (DT). It results in definition of Platelet functional activity index (PFAI) by the following formula:

$$PFAI = ((AD/ADd + AR/ARd + (AD-DD)/AD + (AE-DE)/AE)/4.$$

Induced platelet aggregation is a receptor-mediated process. It is known that the use of each of the inductors makes it possible to determine the sensitivity of a separate group of platelet receptors to proagregants, and to evaluate the functionality of the chain "receptor → platelet activation → platelet aggregation" [5-7]. Most often ADP and adrenaline are used as aggregation inducers. The use of several inductors allows a more extensive test of the sensitivity of platelet receptors and their aggregation response. But due to the fact that part of the receptors can be blocked by inhibitors (both natural and iatrogenic), PFAI of the same patient, calculated using different inducers, can differ significantly. However, the difference between indices calculated for various inducers provides the valuable diagnostic and prognostic information, allowing to assess the state of platelet receptors and the efficiency of antiplatelet therapy. But we do not recommend comparing the indices obtained by various aggregation inducers, as well as various

concentrations of the same inductor.

It is believed that the effect of adrenaline on platelets is significantly weaker in comparison with ADP [17]. Adrenaline causes platelet aggregation without changing their shape, interacting with the alpha-adrenoreceptors of their plasmatic membrane. It is assumed that the mechanism of action of adrenaline is associated with the modulation of membrane properties and changes in their permeability to Ca++ ions.

An important role in platelet aggregation is played by specific receptors belonging to the class P2Y, which react to ADP. By the mechanism of signal transmission, P2 receptors are divided into two families: P2X receptors, which are ligand-dependent ion channels, andP2Y, which belong to the group of G-protein-mediated receptors. Three types of P2 receptors were detected in platelets, i.e. $P2Y_1$, $P2Y_{12}$, and $P2X_1$. Each of them plays a specific role in activation and aggregation of blood platelets [18].

Hence, stimulation of $P2Y_1$ receptors by adenosine diphosphate mobilizes Ca++ ions from depot, leading to modification in the form of platelets and triggering the reversible aggregation of blood platelets. Activation of $P2Y_{12}$ receptors under the action of ADP leads to increased aggregation of both adenosine diphosphate itself and other agonists like collagen, thrombin and epinephrine (adrenaline). Activation of platelets by ADP leads to the expression of receptors for fibrinogen on the plasmatic membrane of platelets. Ions of Ca++ participate in the formation of receptor binding center with fibrinogen. Optimal for binding is the concentration of calcium 0.1-1.0 mmol. The same role is played by Ca++ ions in spontaneous aggregation of platelets [3,17].

Thus, the activity of platelet P2 receptors (G-protein-mediated) and modulation of Ca++ membrane channels can be assessed by induction of ADP aggregation and adrenaline. We believe that this approach, combined with consideration of the disaggregation features, allows us to objectify the researcher's view on the mechanisms of hemostasis unit being studied.

Therefore, each patient received the aggregation test by two inductors, ADP and adrenaline, calculating PFAI as the average value of index with every inductor separately. We consider it significant to provide the calculation examples of PFAI for different patients.

Patient 1 with aggregation test based on ADP induction: AD - 40% AT - 4 min, AR - 54% per min. Additionally defined (Fig. 1): DD - 14%, DT - 5.6 min. Average value of AD in donor

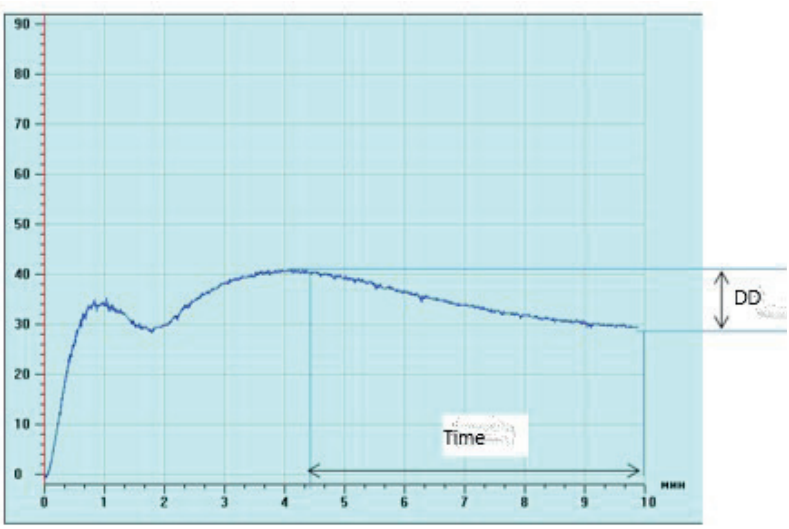

Fig. 1. Evaluation of additional indicators in typical platelet aggregation test of patient #1, where DD is disaggregation degree, and Time is the time for disaggregation

group (control) of 65%, average value of AR in donor group - 60% per min.

Calculation:

40/4=10; AE

14/5.6=2.5; DE

PFAI of Patient 1

PFAI = ((AD/ADd + AR/ARd + (AD-DD)/AD + (AE-DE)/AE)/4 = ((40/65 + 54/60 + (40-14)/40 + (10-2.5)/10)/4 = 0.73

Patient 2 with aggregation test based on ADP induction: AD - 38% AT - 1.5 min, AR - 34% per min. Additionally defined (Fig. 2): DD - 24%, DT - 8 min. Taken into account (see example 1): ADd - 65% and ADr - 60%.

Calculation:

38/1.5=25.33; AE

24/8=3; DE

PFAI of Patient 2

PFAI = ((AD/ADd + AR/ARd + (AD-DD)/AD + (AE-DE)/AE)/4 = ((38/65 + 34/60 + (38-24)/38 + (25.33-3)/25.33)/4 = 0.6

When calculating PFAI for 30 patients, and calculating PFAI as the arithmetic mean of two indices with different inducers, the value of PFAI varied within the range 1.13-0.41, with the Mo = 0.79 mode. Probably, this is the value at which the system is balanced, with a slight predominance of time and speed of aggregation over disaggregation. Because no signs of clinical disturbances were observed we considered the fluctuations of PFAI in the range of 1.13-0.41 as a physiological limits.

While surgical intervention for defects replacement in the clinic, all patients were given two plasma fractions obtained by PRGF method (Endoret Dentistry, BTI Biotechnology Institute, SL, Miñano, Álava, Spain) (Vitoria, Spain) [19]. The protocol for their preparation included the use of special tubes with 0.9 ml 3.8% sodium citrate per 8.1 ml blood, followed by centrifugation (580 g) for 8 minutes at room temperature using a centrifuge for PRGF System IV (BTI, Spain). The resulting plasma was then divided into two fractions: F1 and F2 (as recommended by BTI), then they were separated from each tube using a plasma transfer device PTD2. Fraction F2 was plasma rich in growth factors, i.e. "platelet concentrate" with the highest content of platelets. Fraction F1, poor in platelets, was used to make a fibrin membrane. To activate coagulation and clot formation (and to produce a fibrin membrane), calcium chloride (0.5 ml per 1 ml of plasma) was added to the tubes and desired effect was achieved within 10 min. Prior to activation, samples were taken from fractions F1 and F2 to conduct the analysis for the content

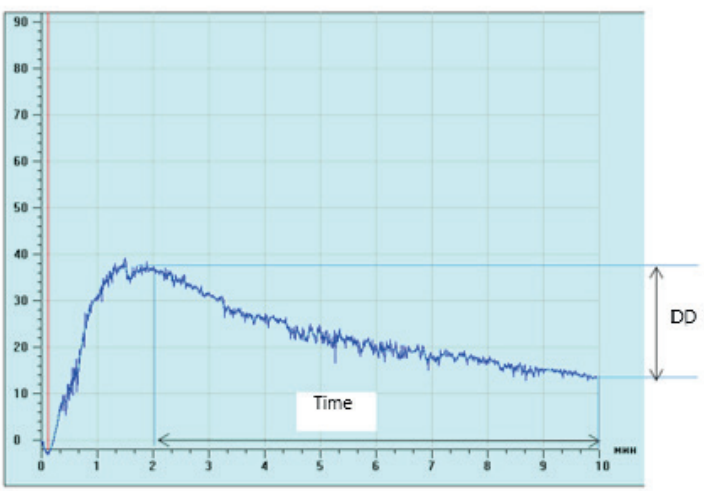

Fig. 2. Evaluation of additional indicators in typical platelet aggregation test of patient #2, where DD is disaggregation degree, and Time is the time for disaggregation

of platelets in them, in addition, the content of other blood elements was determined and the morphology of fibrin membrane was examined.

Clinical study included the analysis of the following parameters: presence or absence of complications associated with the failure or exposure of bone grafts, the purulent and inflammatory processes as well as the inhibition of reparative processes within the post-surgical period. The timing of granulations appearance and complete epithelization of wounds was also identified. The integral result of wound healing was determined using the expert estimation method.

93.3% of patients had a good or satisfactory long term treatment results. Complications in the form of the wound dehiscence and partial destruction of the platelet clot were noted in one patient (3.3%), and their negative consequences were eliminated by conservative methods.

We described platelet content in PRGF EndoRet in our previous work [20]. Their functional properties were also studied depending on the individual hematological parameters of the patient. The inverse relationship was found between aggregation activity of platelets, calculated by PFAI and their amount in fraction F2.

We came to the conclusion that even a small amount of platelets in plasma rich in growth factors is compensated by their increased functional activity, particularly with the aggregation activity. Therefore, the clinical effectiveness of the procedure was almost identical in clinical and laboratory observations, where patients had significantly different platelet properties estimated by the value of PFAI and the content of platelets in the blood also varied.

Based on the fact of existence of such dependence, it can be concluded that there is a biological pattern that ensures the maintenance of blood homeostasis within the limits of physiological fluctuations. Higher content of platelets is accompanied by their low activity towards spontaneous thrombosis, and vice versa, the presence of a reduced amount in the peripheral blood does not lead to bleeding due to their higher aggregation activity.

However, when comparing whole blood PLT of the patient and the functional activity of platelets, determined by aggregation test with the calculation of PFAI, there was no correlation between these values ($r = -0.23$, $p>0.05$).

The number of platelets of plasma (PLT) for all patients was within the normal reference values, with a fluctuation of 174-356 x 10^9/L ($M = 260.76 \pm 44$ x 10^9/L). Hence, patients had no signs of thrombocytopenia or thrombocytosis.

The existence of the biological principle of maintaining the balance of blood homeostasis prompted us to remove from the sample those cases when the properties of platelets hyperaggregation by PFAI combined with high PLT, and the properties of hypoaggregation were accompanied by a low PLT.

Therefore, when evaluating the number of platelets of peripheral blood, PLT< $260x10^9$/l was assumed as a relatively low content in peripheral blood, and PLT≥$260x10^9$/l was conventionally high. We also considered that, the value of PFAI ≥0.8, reflects the high aggregation activity of platelets, and PFAI < 0.8 shows low activity.

Five cases (16%) fell out from the sample, and this exception made it possible to reveal significant inverse correlation dependence. The correlation coefficient was $r = -0.55$ ($p <0.01$).

As a result, it was suggested to determine this homeostasis parameter by simultaneous comparison of the obtained PFAI with PLT, and to consider the normal condition in the presence of low aggregation activity of platelets by PFAI in combination with high PLT, or high aggregation activity by PFAI alongside with low PLT. The hypoactive state is characterized by low aggregation activity on PFAI with low PLT, while hyperactive one showed high aggregation activity on PFAI with high PLT.

For instance, patient #1 had PLT of $306x10^9$/l considered as high by aggregation test. PFAI with ADP = 0.73, with adrenaline = 0.79, integral PFAI = 0.76. Aggregation was defined as low, therefore the condition of the platelet unit

was estimated as normal.

Patient #2 had PLT 253x10^9/l considered as low by aggregation test. PFAI with ADP = 0.6, with adrenaline = 0.58, integral PFAI = 0.59. Aggregation was defined as average, therefore the condition of the platelet unit was estimated as hypoactive.

For practical approbation of the suggested method, the new algorithm for PRGF application was developed, depending on hemostasis platelet unit condition, being identified based on PFAI and PLT (Fig.3).

The F2 fraction was used in accordance to BTI recommendations, if normal condition of hemostasis platelet unit was identified. In hyperactive condition of hemostasis of platelet unit PRGF was used without fractionation because its properties were significantly effective in stimulating the osteogenesis and angiogenesis, and in creating a fibrin matrix as well. When hypoactive state of platelet hemostasis was determined in patient, a double amount of F2 fraction was used to increase the regenerative properties and achieve the desired therapeutic effect.

The use of suggested method in clinical practice made it possible to achieve good or satisfactory results in the absolute majority of patients [20], which proves the validity of the offered approach. Thus such approach can be recommended for the development of therapeutic strategy based on application of platelet protective properties in clinical practice.

CONCLUSIONS

The suggested method for evaluating the functional activity of platelets, by calculating the quantitative index of PFAI, allows to standardize the aggregation test method. Its advantage is to consider the aggregation and disaggregation indicators, the degree and efficiency of these processes, which makes it possible to evaluate the activity of creating aggregates and their strength for a more complete description of the platelets functional properties.

The use of relative indicators, calculated in the group of healthy donors, reduces the influence of subjective factors (operator skill, device calibration, conditions for performing the analysis, etc.) on the result obtained.

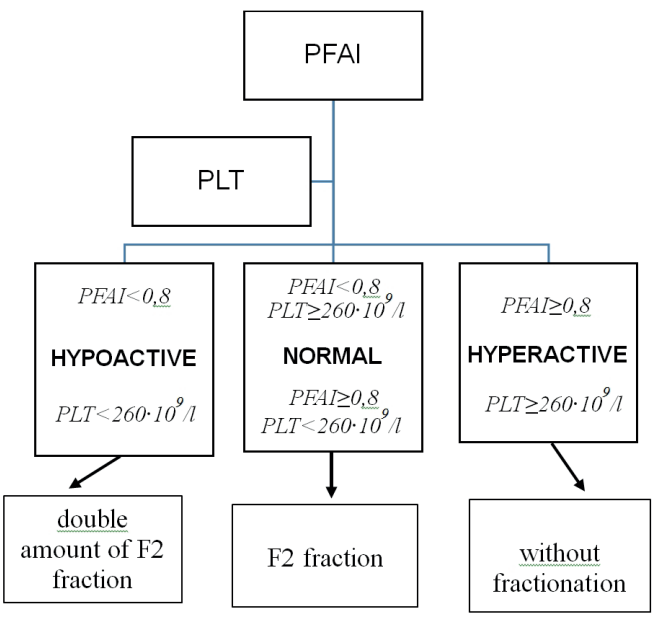

Fig.3. New algorithm for PRGF application, depending on hemostasis platelet unit condition, being identified based on PFAI and PLT. PRGF – plasma, rich in growth factors, PFAI - platelet functional activity index, PLT – platelets. F2 - fraction of plasma rich in growth factors

Evaluation of the platelet hemostasis state on the basis of PFAI and its comparison to PLT is based on the biological principle of maintaining the most important constant of homeostasis. The offered method can be recommended for the development of more effective therapeutic strategies in clinical practice using the reparative potential of platelets.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of coauthors of the article.

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СТАНДАРТИЗАЦИЯ АГРЕГАТОГРАММЫ ДЛЯ ОЦЕНКИ СОСТОЯНИЯ ТРОМБОЦИТАРНОГО ЗВЕНА ГЕМОСТАЗА

Авторы поставили цель разработать и внедрить в клиническую практику способ количественной оценки состояния тромбоцитарного звена гемостаза пациента на основе агрегатограммы. Особенностью способа является анализ параметров прочности созданного сгустка, его дезагрегации и расчёт количественного показателя – индекса функциональной активности тромбоцитов (ИФАТ). Далее, проводилась оценка агрегационных свойств тромбоцитов по ИФАТ, и, в совокупности с их количественным содержанием в периферической крови, характеризовалось состояние тромбоцитарного звена гемостаза как нормальное, гипер- и гипоактивное. На основе указанной характеристики был разработан и внедрен алгоритм использования аутоплазмы, обогащенной факторами роста, при лечении пациентов в клинике челюстно-лицевой хирургии и стоматологии, что позволило у абсолютного большинства получить хорошие или удовлетворительные результаты. Оценка состояния тромбоцитарного звена гемостаза на основе ИФАТ позволяет стандартизировать метод агрегатограммы. Предложенный способ базируется на биологическом принципе поддержания важнейшей константы гомеостаза и может быть рекомендован для разработки более эффективных лечебных походов в практике с использованием репаративного потенциала тромбоцитов.
Ключевые слова: функциональная активность тромбоцитов; дезагрегация; аутоплазма; обогащенная факторами роста.

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СТАНДАРТИЗАЦІЯ АГРЕГАТОГРА МИ ДЛЯ ОЦІНКИ СТАНУ ТРОМБОЦИТАРНОЇ ЛАНКИ ГЕМОСТАЗУ

Автори поставили за мету розробити і впровадити в клінічну практику спосіб кількісної оцінки стану тромбоцитарної ланки гемостазу пацієнта на основі агрегатограми. Особливістю способу є аналіз параметрів міцності створеного згустка, його дезагрегації і розрахунок кількісного показника - індексу функціональної активності тромбоцитів (ІФАТ). Оцінювали агрегаційні властивості тромбоцитів за ІФАТ і, одночасно із їх кількісним змістом в периферичної крові, визначали стан тромбоцитарної ланки гемостазу як нормальний, гіпер- і гипоактивний. На основі зазначеної характеристики був розроблений і впроваджений алгоритм використання аутоплазми, збагаченої факторами росту, при лікуванні пацієнтів в клініці щелепно-лицевої хірургії та стоматології, що дало змогу у абсолютної більшості отримати хороші або задовільні результати. Оцінка стану тромбоцитарної ланки гемостазу на основі ІФАТ дає можливість стандартизувати метод агрегатограми. Запропонований спосіб базується на біологічному принципі підтримки найважливішою константи гомеостазу і може бути рекомендований для розробки більш ефективних лікувальних походів у практиці, яка використовує репаративний потенціал тромбоцитів.
Ключові слова: функціональна активність тромбоцитів; дезагрегація; аутоплазма; збагачена факторами росту.

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Received 20.09.2018