BIOACTIVAREA CITOKINELOR ÎN TUBURI DE PLASTIC DIN PROPILENĂ CU CITRAT DE SODIU 3,2% ÎN VEDEREA DETERMINĂRII CONCENTRAȚIILOR DE TNF-α,TGF ȘI PDGF ÎN DIFERITELE FOTOTIPURI CUTANATE

BIO ACTIVATION OF CYTOKINES USING SODIUM CITRATE 3.2% IN POLYPROPYLENE PLASTIC TUBE TO DETERMINE CONCENTRATE OF TNF-α (TUMOR NECROSIS GROWTH FACTOR ALPHA), TGF(TRANSFORMING GROWTH FACTOR) AND PDGF (PLATELET DERIVED GROWTH FACTOR)IN VARIOUS SKIN PHOTOTYPES

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Autolog platelet concentrate, a therapeutic procedure that is obtained from preparation of platelet rich plasma (PRP), has gained a lot of popularity especially in treatment of diverse aesthetic procedures. The plasma concentrate contains diverse cytokines and growth factors including tumor necrosis growth factors-α (TNF-α), transforming growth factors (TGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF). The active byproduct obtained through this technique play a great role in many processes from stimulating angiogenesis all the way to healing tissue damage.

This study was conducted in order to compare growth factor levels obtained from PRP concentrate from subjects with different skin photo-type: II, III, and IV Fitzpatrick scale. Fresh whole blood was drawn from 20 healthy donors, age from 19 to 42. Plasma was separated by double spin technique, since it results in a higher platelet concentration, activated with Sodium Citrate 3.2% in polypropylene plastic tube. The platelet concentrate was evaluated with enzyme-linked immunosorbent assay (ELISA).

Based on our results, we demonstrated that selected cytokines are present in platelet-rich plasma and that these concentrates of growth factor profile is not influenced by skin photo-type.

Concentratul autolog de trombocite, procedeul terapeutic care se obține din prepararea plasmei bogate în trombocite (PRP), a câștigat multă popularitate, în special în procedurile estetice de tratament. Concentratul plasmatic conține diferite citokine și factori de creștere, incluzând factorii de creștere a necrozei tumorale (TNF-α), factorii de creștere transformatori (TGF), factorul de creștere derivat din plachete (PDGF), factorul de creștere endotelial vascular (VEGF) și factorul de creștere epitelial (EGF). Produsul activ obținut prin această tehnică joacă un rol important în multe procese, de la stimularea angiogenezei până la vindecarea leziunilor tisulare.

Scopul acestui studiu a fost de a compara nivelele factorilor de creștere conținute în produsul PRP concentrat, obținut de la subiecți cu diferite foto tipuri de piele conform scalei Fitzpatrick: II, III și IV. Sângele integral proaspăt a fost extras de la 20 de donatori sănătoși, cu vârsta cuprinsă între 19 și 42 de ani. Plasma a fost separată prin tehnica de centrifugare dublă, având drept rezultat o concentrație mai mare de trombocite, activată în tuburile de plastic de polipropilenă cu citrat de sodiu 3,2%. Concentratul trombocitar a fost evaluat cu ajutorul testului ELISA. Pe baza rezultatelor noastre, am demonstrat că citokinele selectate sunt prezente în plasma bogată în plachete. De asemenea, am evidențiat faptul că nivelele acestor molecule nu sunt influențate de fototipul pielii.

Keywords: *growth factors, cytokines, enzyme-linked immunosorbent assay (ELISA), Platelet-Rich Plasma (PRP)*

1. Introduction

The interaction of diverse cytokines and growth factors with the adhesion molecules stimulates the pro-inflammatory processes, cellular proliferation and differentiation and boosts extracellular matrix which have a great impact on tissue regeneration. Platelet rich plasma (PRP), a biological active product, is the concentrations of platelets from a volume of plasma. These platelets include a number of distinct cytokines: tumor necrosis factors α(TNF-α), transforming growth factors (TGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF), among others. [1,2] By virtue of high concentration of these growth factors, protein rich plasma preparations is used in a broad range from diverse surgical methods all the way to aesthetic treatments. [3]

The biological action of these growth factors, which bind to a specific receptor on the cellular surface, is precise. Transforming growth factor alpha/beta (TGF α/β) stimulates secretion and synthesis of collagen. Platelet derived growth factor (PDGF) has

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a mitogenic effect on mesenchymal cells, acts on osteoblasts and fibroblasts which leads to regulation of collagen secretion. Tumor necrosis factor α(TNF-α) is a pro-inflammatory cytokine which stimulates the adhesion molecules, activates and increases permeability, and works on angiogenesis and the immune response. Vascular endothelial growth factor (VEGF), secreted by platelets and endothelial cells, play an important role in angiogenesis and vessel permeability. The epithelial growth factor (EGF) stimulates epithelial cellular functions and triggers angiogenesis and collagen secretion.[4,5]

The aim of this study was to detect and quantify the amount of diverse growth factors and cytokines in PRP concentrates in diverse skin photo-type of healthy young individuals using enzyme-linked immunosorbent assay (ELISA). The platelet rich plasma was prepared by using a double spin method and then activated with sodium citrate that stimulates platelet degranulation and the release of growth factors.[6 - 8].

2. Materials and methods

The study includes 20 healthy subjects, six males and fourteen females, age from 19 to 42 years. The patients have skin photo-type ranging from type II to type IV, according to Fitzpartick criteria scale classification. The selection criteria were as following: healthy 18 years of age or older participant, willing to actively participate in the study, and a written consent approved by the ethics committee.

From the healthy volunteers 20ml of whole blood was collected in Sodium Citrate 3.2% polypropylene plastic tubes with a volume ratio of . The samples were mixed and centrifuged using the double spin method of platelet separation. In the first step, the centrifugation was done at a speed of 900g for 9 minutes. The supernatants were transferred to new sterile polypropylene plastic tubes and centrifuged a second time at a speed of 1400g for 14 minutes [9 - 13].

After centrifugation, the PRP samples were stored at -80◦C until they were used. Water baths was used to thaw the PRP samples in a vacuum sealed wrap in order to protect from bacterial contamination. An average time for thawing was about 20 minutes. TNF-α, TGFβ1 and FGF2 levels were assessed by enzyme-linked immunosorbent assay, using Abbexa ELISA kit (TNF-α, TGFβ1) and, MyBiosource (FGF2), following the manufacturer's instruction. Briefly, after standard preparation,[14] 100µl of samples were tested. The absorbance was measured at 450nm and the concentration of cytokines was calculated according to the calibration curve. We utilised the following concentrations value for the standard curve: TNF-α (100-50-25-12,5-6,25-3,125-1,5625 pg/ml), TGFβ1 (1000-500-250-125-62,5-31,2-16,625 pg/ml) and, FGF2 (1000-500-250-125-62,5-31,2-16,625 pg/ml). R^2 value was 0,9909 for TNF-α, 0,9906 for TGF β1 and, 0,9902 for FGF2. Intra and Inter assay variability was less than 10%.

3. Results

In our healthy volunteer study we highlighted three cutaneous photo-types (II, III and IV), characteristic for our geographic area. The average age of patients with skin- phototype II was 25.5±5.95, for subjects with photo type III was 34.33±5.71 and 30.66±8.38 for subjects with phototype IV. We examined the biological parameters (leukocytes, platelets, TNF-α, TGF β1 and FGF2) for each of those phototypes.

Before platelet separation stage of PRP, the number of cells involved in producing cytokines (leukocytes and platelets) was analysed. The obtained leukocyte count for skin phototype II was 7.98 $x10^3$/µL ±1.21, 7.41 $x10^3$/µL ± 1.35 for phototype III and 7.17$x10^3$/µL ± 0.7 for skin phototype IV.

The mean platelet count, which were all within the range of normal biological values, was 378.33 $x10^3$/µL ± 39.11 for skin phototype II, 353 $x10^3$/µL ± 48.36 for skin phototype III and 389 $x10^3$/µL ± 94.79 for skin phototype IV (Table 1). An average of1.95ml (1.5-2.2 ml) was obtained from the PRP concentrate which are demonstrated in Table 2.

Tabel 1

Analysis of whole blood cells (mean± SD)
Analiza numărului total de celule sanguine

Blood cells / Skin type after Fitzpatrick	Leukocytes ($x10^3$/µL)	Platelet (x103/µL)
II	7.98±1.21	378.33± 39.11
III	7.41± 1.35	353 ± 48.36
IV	7.17 ± 0.7	389 ± 94.79

Table 2

PRP volume after centrifugation in polypropylene plastic tube
Volumul de PRP obținut după centrifugare în tuburile de polipropilenă

Parameters / Skin type after Fitzpatick	Volume PRP (ml) (mean±SD)
II	2.03±0.1
III	1.9±0.2
IV	1.9±0.1

Concentration (pg/ml) of TNFα from PRP was 8.285±2.14 in patients with skin photo-type II, 8.55±1.09 in patients with skin photo-type III, and 7.38±2.7 in patients with skin photo-type IV. Obtained concentration of TGFβ was 0.34±0.08 in patients with skin photo-type II, 0.36±0.04 in patients with skin photo-type III, and 0.31±0.03 in patients with skin photo-type IV. The FGF2 levels of PRP was 0.11±0.04pg/ml in skin phototype II, 0.105±0.04 pg/ml in skin photoype III, and 0.108±0.03 pg/ml in skin phototype IV. There was

also a significant correlation between the age of patient and the number of leucocytes present (r = -.463, p = .053, two-tailed (Tabel.3), decreasing number of leukocytes with increasing age(Fig 1). We have not noticed any other significant difference in the concentrations of the studied cytokines according to the skin photo-types(Fig 2). The outcome of this study display that the relationship between cellular components and cytokine concentration is basically dependent on the cytokine type.

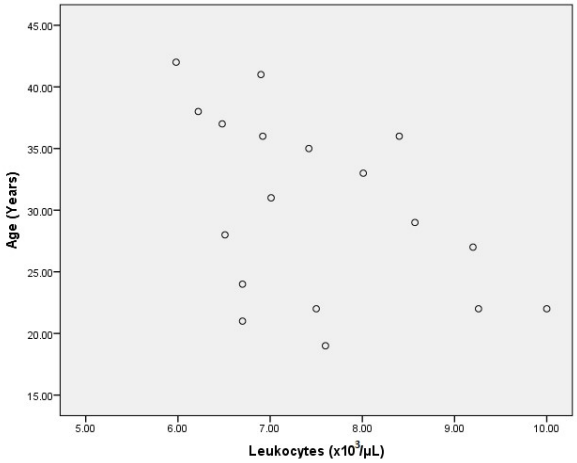

Fig. 1 - Correlation between age and leukocytes.
Corelația între vârsta subiecților și numărul de leucocite.

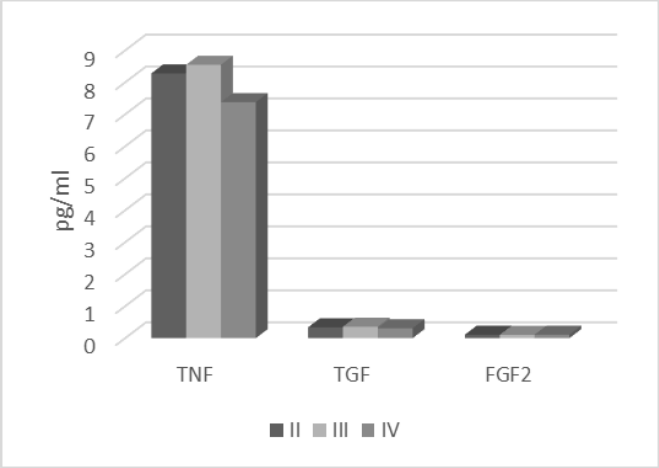

Fig 2 - Concentration of cytokines according to skin phototype.
Concentrația citokinelor în funcție de fototipul cutanat.

4.Discussion and conclusion

Numerous growth factors, from activated platelets, are key mediators of wound healing and tissue regeneration. Various in vitro studies reported beneficial effect of these growth factors obtained from PRP preparations. [6,15,16] Protein rich plasma applied on fibroblasts cultures showed to increase fibroblast matrix protein synthesis, fibroblastic proliferation and collagen expression protein. [12,17] Other studies demonstrated stimulation and differentiations of osteoblast activity resulting in bone healing and regeneration.[18] Autologous protein rich plasma is a completely safe procedure that doesn't create any adverse actions or complications.

The purpose of this study was to determine the quantitate analysis of major cytokines (TNFα, TGFβ si FGF2) in PRP preparation obtained by double spin method activated with Sodium Citrate 3.2% in polypropylene plastic tube. Simultaneous we wanted to evaluate and observe variation in cytokines level with skin photo-type. The regenerative potential of PRP concentrates is

Table 3

Analysis of studied cytokines and clinico-biological parameters / *Analiza corelației nivelurilor de citokine studiate și parametrii clinicobiologici*

		Age	Leukocytes	Thrombocytes	TNFa	TGFb	FGF2
Age	Pearson Correlation	1	-.463	-.124	.096	-.002	.399
	Sig. (2-tailed)		.053	.623	.704	.994	.101
	N	18	18	18	18	18	18
Leukocytes	Pearson Correlation	-.463	1	-.145	.161	.392	.059
	Sig. (2-tailed)	.053		.565	.522	.107	.817
	N	18	18	18	18	18	18
Thrombocytes	Pearson Correlation	-.124	-.145	1	-.154	.068	.097
	Sig. (2-tailed)	.623	.565		.543	.790	.702
	N	18	18	18	18	18	18
TNFa	Pearson Correlation	.096	.161	-.154	1	.144	.364
	Sig. (2-tailed)	.704	.522	.543		.569	.138
	N	18	18	18	18	18	18
TGFb	Pearson Correlation	-.002	.392	.068	.144	1	.144
	Sig. (2-tailed)	.994	.107	.790	.569		.569
	N	18	18	18	18	18	18
FGF2	Pearson Correlation	.399	.059	.097	.364	.144	1
	Sig. (2-tailed)	.101	.817	.702	.138	.569	
	N	18	18	18	18	18	18

directly proportional to the type and concentrations of growth factors. However, there are differences in the status of cytokines, individual or demographic, which is reflected in the therapeutic effect of PRP. [19].

Information from specialized literature on this matter is reduced. Most studies aimed at highlighting the levels of growth factors depending on the number of blood cells or the method of preparation. In our study we did not find a significant correlation between the number of white blood cells, platelets and the level of cytokines studied. Also, concentrations of TNFα, TGFβ and FGF2 were relatively the same for all three skin photo-types studied. Roh *et al*. analyzed cytokine release kinetics at different time intervals and in the presence or absence of activation factors. The study found that the level of cytokines differs depending on the method use to obtain the PRP, as well as the type of activator used and the activation time. [20]

The levels of TGFβ and FGF2 are determined by the acquiring procedure and do not depend on the number of platelets. Some studies reported values that could be measured in ng/ml [21], however in our study the values obtained, regardless of skin type, were much lower. Similar results were also reported by the study by Pochini et al. Values obtained for FGF2 were similar to reports from other similar studies, being undetectable or very low. [21]

TNFα values in PRP concentrates were superior to other studied cytokines. TNFα is a proinflammatory cytokines that plays an important role in stimulating the synthesis of other types of cytokines. Shukla and Patel suggested that TNFα level is induced by WBC (leukocytes) and its concentration is related to storage level. [22]

Platelet rich plasma contain a high concentrations of growth factors. These growth factors concentration released from activated platelets, activated by Sodium Citrate 3.2% in polypropylene plastic tube implies that protein rich plasma (PRP) is a hopeful participant in promoting tissue healing and regeneration whether it's use for medical or aesthetic procedures. The prevalence of female subjects in our study (16f/4m) was due to the fact that, women are more prone to aesthetic procedures related to PRP. The reduced number of participants is a limitation of our study but a base for further studies.

We demonstrated that activated factors in PRP product is not dependent on skin phototype at levels similar to other studies, suggesting that other factors in time contribute to the imbalance of those cytokines that reflect in aging of the skin or even franc pathologic conditions.

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