Cytokine, chemokine, and growth factor profile of platelet-rich plasma

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Abstract (150-400 words unstructured)

During wound healing, biologically active molecules are released from platelets. The rationale of using Platelet Rich Plasma (PRP) relies on the concentration of bioactive molecules and subsequent delivery to healing sites. These bioactive molecules have been seldom simultaneously quantified within the same PRP preparation. In the present study, the flexible Bio-Plex system was employed to assess the concentration of a large range of cytokines, chemokines and growth factors in sixteen healthy volunteers so as to determine whether significant baseline differences may be found. Besides IL-1b, IL-1ra, IL-4, IL-6, IL-8, IL-12, IL-13, IL-17, INF-γ, TNF-α, monocyte chemoattractant protein-1 (MCP-1) (CCL-2), MIP-1a (CCL-3), RANTES (CCL-5), bFGF, PDGF, VEGF that were already quantified elsewhere, the authors reported also on the presence of IL-2, IL-5, IL-7, IL-9, IL-10, IL-15 G-CSF, GM-CSF, Eotaxin (CCL-11), CXCL10 chemokine (IP-10), MIP 1b (CCL-4). Among the most interesting results, it is convenient to mention the high concentrations of the HIV-suppressive and inflammatory cytokine RANTES and a statistically significant difference between males and females in the content of PDGF-BB. These data are consistent with previous reports pointing out that gender, diet and test system affect the results of platelet function in healthy subjects, but seem contradictory when compared to other quantification assays in serum and plasma.

The inconsistencies affecting the experimental results found in literature, along with the variability found in the content of bioactive molecules, urge further research, hopefully in form of Randomized Controlled Clinical Trials, in order to find definitive evidence of the efficacy of PRP treatment in various pathologic and regenerative conditions.

Introduction

Platelet-rich plasma (PRP) emerges amongst the most innovative autologous blood products used to enhance tissue healing and regeneration. Blood withdrawn from a patient's peripheral vein is centrifuged to concentrate platelets. PRP may be immediately used either as it is, or in combination with other biomaterials. For sake of precision, based on leukocytes and fibrin content, PRP products are subdivided as follows: pure platelet-rich plasma (P-PRP) also known as plasma rich in growth factors (PRGF), leukocyte- and platelet-rich plasma (L-PRP), pure platelet-rich fibrin (P-PRF), and leukocyte- and platelet-rich fibrin (L-PRF) [1]. Usually in the form of gel or liquid, P-PRP and L-PRP have both a low-density fibrin network, while P-PRF and L-PRF, available only in the gel form, contain high-density fibrin network.

Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF-I), transforming growth factor β-I (TGFβ-I), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) are just a few of the growth factors (GFs) present within the α-granules of platelets [2,3]. Such a wide range of GFs is one of the keys to understand the multifaceted roles of PRP, including proliferation and differentiation of cells belonging to the musculoskeletal and vascular systems [4]. However, α-granules also contain different interleukins (ILs) and chemokines, such as IL-1 β, IL-8, and MIP-1-2-3 [5,6], that are inflammation mediators able to stimulate cell chemotaxis and maturation.

Several studies [7–9] evaluated in vitro the effect of PRPs supporting their ability to improve proliferation and osteogenic activity of osteoblasts and mesenchymal stem cells. However, these effects were dependent on the PRP composition [10]. Somehow data become controversial when in vivo settings are compared, since opposite outcomes are equally available in the scientific literature in favor [11–21] or against [19,22–27] the addition

of PRP to biologic and synthetic graft materials for bone regeneration purposes. Interestingly, the association of PRP with mesenchymal stem cells showed mostly satisfying results [28,29] in the field. Recently, regenerative medicine and tissue engineering focused on the use of GFs [30] and cell-based therapy to improve the quality and speed of healing, extending the PRP application from the traditional dental and maxillofacial field to the treatment of musculoskeletal injuries [31].

Currently, on the market, there exist more than 40 commercial systems able to concentrate whole blood into a platelet-rich substance. Such a relevant number of different preparation protocols should be taken into account whenever inconsistencies are found from comparing clinical outcomes reported in the literature [32]. The quality of PRP could be affected by patient's heterogeneity in terms of age, gender, body mass index, comorbidities, healing capabilities, and different lifestyles [33]. In order to reduce at least the protocol dependent variability, the authors selected a closed preparation system, which, being automatic, limits the possibility of operator dependent errors and reduces the risk of microbial contamination, while processing the blood.

The aim of the present research is to assess the concentration of a large range of cytokines, chemokines and growth factors in a proper sample of healthy volunteers so as to determine whether significant baseline differences may be found.

Material and methods

Sample preparation

Sixteen healthy volunteers (mean age= 24.25±4.96 years, age range: 20 to 40 years, 10 females and 6 males) were recruited as possible blood donors, based on their medical histories, at the Interdepartmental Research Center (IRC) Dental School of the University of Turin, between January 1st and February 1st 2015. The study protocol was approved by the Ethics Committee of the IRC. The informed consent was obtained. PRP samples were prepared with the CPunT System, (Eltek Group, Casale Monferrato, Italy). The CPunT Preparation System is a closed system medical device that consists of a main unit, which separates the PRP from other substances, and the appropriate disposable components (for detailed information please refer to supplementary materials). Briefly, the samples were processed according to the following protocol. First, blood was withdrawn and centrifuged at 1200 rpm for 11 minutes in an ELTEK centrifuge (Cat number 10714800), after adding ACD (Sodium Citrate / Citric Acid / Glucose SALF SpA, Italy). This centrifugation separated the buffy coat and the red blood cells from platelets and plasma, which were automatically pushed into a separate bag under the control of an optical sensor so as to prevent any buffy coat contaminations. The bag was subsequently positioned in the centrifuge basket for a second centrifugation (2000 rpm for 9 min). At this step, the Platelet Concentrate (PC) was sedimented on the bottom of the bag. Part of PPP (Poor Platelets Plasma), 4 mL, was removed through the accessory 10-mL syringe with needle. The bag with Platelet Concentrate and the remaining plasma were mixed gently, massaging the bag. Thus, the Platelet Rich Plasma (PRP) was obtained.

Detection of Interleukins, Chemokines and Growth Factors using Bio-Plex system

The PRP samples thus obtained were characterized by measuring the concentration of the following specific biomolecules: interleukin-1b, interleukin 1ra, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-12, interleukin-13, interleukin-15, interleukin-17, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-gamma (INF-γ), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), CXCL10 chemokine (IP-10), MIP-1a, MIP 1b, RANTES, eotaxin, platelet derived growth factor (PDGF), basic-fibroblastic growth factor (bFGF), vascular endothelial growth factor (VEGF). The flexible Bio-Plex system (Bio-Rad Laboratories, Hercules, CA, USA) was emplyed. All samples were analysed following the manufacturer's protocol. At least two independent repetitions in duplicate were made per sample. Concentrations of the analytes were expressed in pg/ml. A standard curve ranging on average from 0.15 pg/ml to 3700 pg/ml (High Photomultiplier Tube Setting -PMT setting) was prepared and then fitted by Bio-Plex Manager software.

Blood count

Platelets from every processed sample were counted with Neubauer Improved counting chamber (Marienfeld, Lauda-Königshofen, Germany).

Statistical Analysis

Red blood cells and platelet count were analyzed using Student t test. A descriptive analysis of Bio-Plex data was performed presenting data using means±standard error

mean (SEM). Differences between groups were analyzed using the two-way ANOVA with Tukey's multiple comparison test.

All the statistical analysis were performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) and were conducted with a 0.05 level of significance.

Results

The blood processing work-flow used to get PRP is portrayed in Fig.1 along with the number of the platelets at the key steps of the procedure. The concentrations of the Cytokines (Fig.2), Chemokines (Fig.3) and Growth factors (Fig.4) detected within the PRPs of the 16 healthy volunteers are reported as means and are subdivided by gender. It is noteworthy that only PDGF differed in a statistically significant way between males and females.

Discussion

Platelets are fundamental factors of the clotting cascade that is central to the wound healing [34]. During this process, biologically active molecules are released from the α-granules [5,35]. Within the first 10 minutes after activation about 70% of the GFs are secreted reaching almost 100% within the first hour [36]. The rationale of the use of PRP is that it concentrates more platelets than the whole blood, allowing the delivery of bioactive GFs and molecules that promote tissue healing. To date, however, there is still little information available about chemokines, cytokines and growth factors simultaneously quantified within the same PRP preparation. This lack of knowledge may be due to the analytic techniques used, which are mostly ELISA-based kits [6].

To address the issue, the flexible Bio-Plex system was employed in the present study. Based on a capture sandwich immunoassay, this technology allowed for the simultaneous dosage of different biomolecules to be done in a single microplate well, as previously described [37]. Besides the biomolecules that had already been quantified in PRPs such as: IL-1b IL-1ra [38,39], IL-4 [40], IL-6 [39], IL-8 [40,41], IL-12 [6], IL-13 [40], IL-17 [40], INF-γ (detected contrary to [40]), TNF-α [39,40], monocyte chemoattractant protein-1 (MCP-1) (CCL-2) [42], MIP-1a (CCL-3) [42], RANTES (CCL-5) [6], bFGF [6], PDGF [6,38,41], VEGF [6,38,41] the authors reported also on the presence of IL-2, IL-5, IL-7, IL-9, IL-10, IL-15 G-CSF, GM-CSF, Eotaxin (CCL-11), CXCL10 chemokine (IP-10), MIP 1b (CCL-4).

Some considerations immediately emerge when analyzing the results here shown. Although RANTES (CCL5) plays an active role in recruiting leukocytes into inflammatory sites, its more refined modulatory and chemotactic properties have been already shown by El-Sharkawy et al [6]. The high concentration of RANTES here detected is therefore consistent with the acceleration of healing and regenerative processes often favorably reported. Interestingly, from the data analysis, the only difference concerning possible

gender variability dealt with the concentration of PDGF, which was higher in males, independently of the platelet count. This result may be partially discordant with the data published by Gomez et al.[43], but it is certainly consistent with Miller and colleagues [44], who pointed out that gender, diet and test system affected the results of platelet function in healthy subjects. It is noteworthy that the values of PDGF-BB here described are quite similar to those reported previously [6], while the content of PDGF was respectively higher in serum [45] and lower in plasma [46] according to other studies. Contrary to Amable et al. [40], INF-γ could be detected and quantified in non irrelevant amount. Also, other authors described more elevated PDGF concentrations in plasma than we could determine within PRPs. [47]

These inconsistencies affecting the experimental results found in literature may owe to countless factors such as the varied types of activating methods (CaCl2, thrombin, batroxobin, bovine thrombin, and thrombin added to CaCl2), the different sample volumes of blood (from 9 to 120 mL) or PRP (from 3 to 32 mL), the number of spins during centrifugation (1 or 2), and the platelet concentration (from 1x to 18x) [48,49].

The widely recognized importance of PRP as an accessible source of growth factors is supported even by this study portraying the variety of bioactive molecules. Indeed, it must not be neglected that, besides the mitogenic and anabolic properties individually attributed to PDGF-BB, FGF-b, VEGF and other well known growth factors, synergistic effects may be taken into consideration also in case of the relatively low concentrations of signal molecules whose role is less characterized [50]. In conclusion, further research, hopefully in form of Randomized Controlled Clinical Trials, is required to find definitive evidence of the efficacy of PRP treatment in various pathologic and regenerative conditions.

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