Biological Characterization and In Vitro Effects of Human Concentrated Growth Factor Preparation: An Innovative Approach to Tissue Regeneration

Elisa Borsani[1#], Veronica Bonazza[1#], Barbara Buffoli[1], Marco Angelo Cocchi[1], Stefania Castrezzati[1], Giorgio Scarì[2], Francesco Baldi[3], Stefano Pandini[3], Stefano Licenziati[4], Silvia Parolini[5], Rita Rezzani[1] and Luigi F Rodella[1*]

[1]Department of Clinical and Experimental Sciences, Division of Anatomy and Physiopathology,, University of Brescia, Viale Europa n.11 , 25123 Brescia, Italy
[2]Department of Biosciences, University of Milan, Via Celoria n. 26, 20133 Milano, Italy
[3]Department of Mechanical and Industrial Engineering, Via Branze n. 38, 25123 Brescia, Italy
[4]S.T.E.M. Laboratorio Analisi Cliniche Srl, Via Rodi n. 49, 25121 Brescia, Italy
[5]Department of Molecular and Translational Medicine, Division of Experimental Oncology and Immunology, University of Brescia, Viale Europa n. 11, 25123 Brescia, Italy
#The Authors share the first authorship

Summary

Scientific background: Platelet concentrates are nowadays widely applied in different clinical fields to improve soft tissue and bone regeneration. "Concentrated Growth Factors" (CGF) is a new generation of platelet concentrate products, which exhibits an interesting clinical and biotechnological application potential.

Aim of the study: The aim of this study is to assess the biological rationale for the use of CGF, by evaluating blood cell localization, the *in vitro* cumulative release of seven growth factors (PDGF-AB, VEGF, TNF-α, TGF-β1, BDNF, BMP-2 and IGF-1), its *in vitro* effects on cell proliferation and its mechanical behavior.

Methods: CGFs were obtained from volunteer donors. Blood cell localization was evaluated after properly morphological staining and immunohistochemistry. The amount of growth factors release was measured at 5 hours, 1, 3, 6, 7 and 8 days, using ELISA assay. Cells were cultured with and without CGF and their proliferation was evaluated after 72 hours, performing the quantification of Ki-67, using flow cytometry (FACS). The mechanical response of CGF under compression was also attempted.

Results: The results showed that platelets and leukocytes were found in a very thin space called "buffy coat", localized between the white and red part of CGF. Each growth factor evaluated, had a specific kinetic release with a great variability among subjects. The *in vitro* cell proliferation was stimulated. CGF showed an "apparent plasticity" and its mechanical response was influenced by fibrin network structure.

Conclusion: These findings support the CGF's clinical use and will allow us to better understand and improve the clinical outcomes.

Keywords: Concentrated Growth Factors (CGF); Human; Regeneration; Blood; Platelets

Abbreviations: A_0: Cross-Section; BDNF: Brain Derived Growth Factor; BMP: Bone Morphogenetic Proteins: CGF: Concentrated Growth Factors: EGM: Endothelial Growth Medium:EBM-2: Endothelial Basal Medium 2; F: Load; FGM: Fibroblast Growth Medium; FBM: Fibroblast Basal Medium; H: Height; H_0: Initial Height; HE: Hematoxylin And Eosin Staining; HOB: Human Osteoblasts; HRP: Horseradish Peroxidase; HUVEC: Human Umbilical Vein Endothelial Cells; IGF: Insulin-Like Growth Factor; MGG: May-Grünwald-Giemsa Stain; NHDF: Normal Human Dermal Fibroblasts; OBM: Osteoblast Basal Medium; OGM: Osteoblast Growth Medium; PDGF: Platelet Derived Growth Factor; PPP: Platelet Poor Plasma; PRF: Choukroun's Platelet Rich Fibrin; PRP: Platelet-Rich Plasma Preparations; RBC: Red Blood Cells; RP: Red Part; WP: White Part; BC: Buffy Coat; SEM: Scanning Electron Microscopy; TGF: Transforming Growth Factor; TNF: Tumor Necrosis Factor; V: Volunteer; VEGF: Vascular Endothelial Growth Factor; WB: Whole Blood; Σ: Nominal Stress; E: Nominal Strain.

Introduction

"Concentrated Growth Factors" (CGF) is one of the several types of platelet-rich plasma preparations (PRP) developed to date [1] and represents a new generation of PRP, which exhibits an interesting clinical and biotechnological potential. PRPs are defined as preparations with a high concentration of platelets in a small volume of plasma [2], containing also growth factors, leukocytes and fibrin matrix [3-7]. The strength of PRP is in the autologous technology to obtain growth factors [8,9] released from platelets in order to promote tissue regeneration [10]. These 100% autologous preparations not only enhance tissue healing, but also improve the clinical outcomes of various surgical procedures, reducing complications such as pain, inflammation and morbidity [11].

In regenerative medicine, three factors are important to optimize the regenerative process: scaffold (biological, natural or synthetic), growth factors and autologous cells. All of these are present in CGF developed by Sacco in 2006. CGF, in its solid form, is obtained by centrifuging of blood samples collected in vacuum tubes, using a special centrifuge device (Medifuge, Silfradent srl, Italy), similar to Choukroun's platelet rich fibrin (PRF) [12-17]. CGF technology has an interesting

characteristic: i.e. the easy and speedy one-step preparation of a larger, denser and richer in growth factors fibrin matrix than the other solid PRPs. Using Scanning Electron Microscopy (SEM) analysis, Rodella and colleagues [18] showed the presence of a fibrin network constituted by thin and thick fibrillar elements with multiple platelets trapped among the fibrin network. We can therefore say that it represents an optimal autologous scaffold. Nevertheless, even if mechanical characterization of some PRPs has been reported in Literature to better understand their potential applications [19,20], no data are available about CGF.

The growth factors, are a class of natural biological mediators that regulate key cellular events in tissue repair, including cell proliferation, differentiation and extracellular matrix synthesis. Platelet activation and degranulation causes the release of a large number of biological factors, including Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor (IGF), Transforming Growth Factor (TGF), Tumor Necrosis Factor (TNF), Brain Derived Growth Factor (BDNF) and Bone Morphogenetic Proteins (BMP) [21-24]. The presence in CGF of TGF-β1and VEGF has been reported by our research group [18]. Previous studies have demonstrated that local application of growth factors alone or mixed with bone allograft increased bone growth, by accelerating healing of soft tissues and facilitating periodontal ligament repair in both animal and human studies [25].

The presence of autologous cells such as platelets and leukocytes, including the CD34 positive cells, have been described [18]. Increasing evidences point to the role of circulating CD34 positive cells [26,27] in vascular maintenance, neovascularization and angiogenesis [28,29]. The presence of these cells in PRP preparations, promotes tissue regrowth [30].

CGF seems to possess a good regenerative capacity and versatility. For example, it has been reported that CGF has a positive effect for the following: sinus and alveolar ridge augmentation [31]; pre-implant augmentation procedures [32]; promotion of *in vitro* proliferation, osteogenic maturation and mineralization of mesenchymal stem cells and healing of critical-size bone defects *in vivo* [33]; promotion of *in vitro* periodontal ligament stem cells proliferation [34], management of chronic venous ulcers [35]. Considering the small amount of data on morphology, biological properties and regenerative potentiality of CGF, the aim of this study was to evaluate the blood cell localization, the *in vitro* cumulative release of growth factors and the *in vitro* power of growth on three different types of human cell lines: the Normal Human Dermal Fibroblasts (NHDF), the human umbilical vein endothelial cells (HUVEC) and the Human Osteoblast (HOB). Furthermore, a mechanical characterization of CGF was also performed by means of compression tests.

Materials and Methods

All experiments were conducted at the Laboratory of Anatomy and Physiopathology, Department of Clinical and Experimental Sciences, of the University of Brescia, between June 2014 and April 2015.

Blood collection

For the experiments, the venous blood was collected by piercing a superficial vein with a 21-gauge needle from 3 healthy adult volunteers of Caucasian ethnicity consisting of 1 men (V1) and 2 women (V2,V3), aged 28 to 39 years and with platelets, red blood cells and leukocytes levels within the normal range. Exclusion criteria included: systemic disorders, smoking, infections, non-steroidal anti-inflammatory drug use, an hemoglobin level <11 g/dl for females and <13.5 g/dl for males.

The collected samples were always processed immediately after blood sampling. The research was conducted according to the principles of the Declaration of Helsinki.

Blood analysis

The subjects underwent to a hematologic blood test (erythrocytes, leukocytes and platelets) performed by laboratory S.T.E.M. analysis (Brescia, Italy) to recruit patients with normal hemochrome values, specified in inclusion criteria.

CGF preparation

The CGF was produced as follows: 9 mL of blood was drawn into each sterile Vacuette tube (Greiner Bio-One, GmbH, Kremsmunster, Austria) silicon coated as a serum clot activator. These tubes were then immediately centrifuged in a special machine (Medifuge MF200, Silfradent srl, Forlì, Italy) using a program with the following characteristics: 30 seconds acceleration, 2 minutes at 2,700 rpm, 4 minutes at 2,400 rpm, 4 minutes 2,700 rpm, 3 minutes at 3,000 rpm and 36 seconds deceleration and stopped. At the end of the process, three blood fractions were identified: (1) the upper layer, representing the liquid phase of plasma named platelet poor plasma (PPP), (2) the lower layer, at the bottom of the tube, consisting in free red blood cells (RBC); (3) the middle layer, representing the solid CGF, consisting in three parts: the upper white part (WP), the downer red part (RP) and the middle "buffy coat" (BC), interface between white and red part (Figure 1).

After centrifugation, CGF was removed from each tube, using sterile tweezers and placed on the surface of sterile petri dish, under a laminar flow cabinet. The solid CGF was obtained by cutting and discarding the lower fraction of the red part of CGF, 0.5 cm under the white part. Subsequently, each CGF was processed in relation to the experimental protocols.

The biological experiments were performed in triplicate to ensure repeatability of results, so each volunteer underwent a blood collection at different time-periods, for a total of 3 tubes for morphological staining and immunohistochemistry, 18 tubes for cumulative growth factor release and 18 tubes for each cell line cultures. The mechanical tests were carried out on 1 sample for each volunteer.

Histomorphological analysis

Immediately after centrifugation the CGF was collected and fixed in 10% buffered formalin for 24 hours, embedded in paraffin according to standard procedures and cut at 8 μm by a microtome (Microm HM 325). Histomorphological assessment of CGF was made with two histological stain: the May-Grünwald-Giemsa (MGG; Bio-Optica, Milan, Italy) and the Hematoxylin and Eosin staining (HE; Bio-Optica, Milan, Italy). The stains were performed according to the manufacturer's protocol. However, with these stainings, the platelets were not clearly identifiable and for this reason CD61 immunohistochemistry, a platelet marker was also performed.

Platelet immunohistochemistry

Alternate paraffin sections were processed by immunohistochemistry. The sections were deparaffinised, rehydrated and subjected to antigen retrieval in 0.05M sodium citrate buffer (pH 6.0) in hot water bath (98°C for 20'). Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide. Sections were immunestained with the monoclonal antibody anti-human CD61 (platelet membrane glycoprotein IIIa, dilution 1:50,
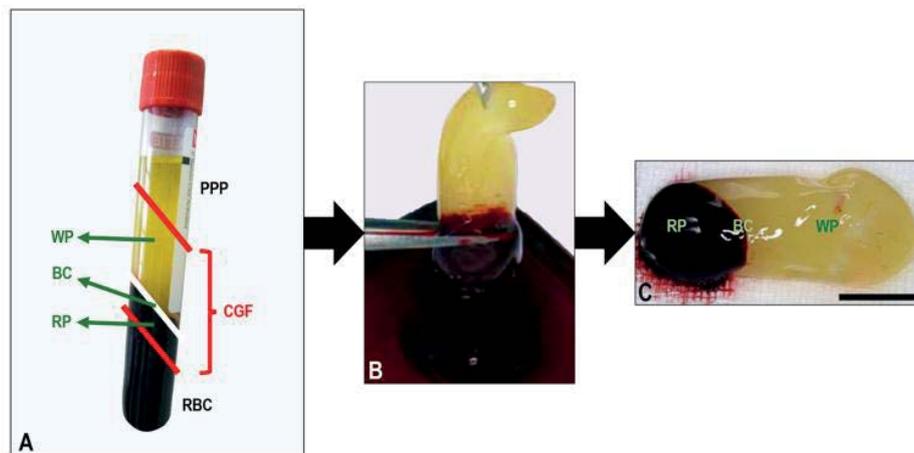
Figure 1: Blood sample after CGF protocol of centrifugation. Three layers are obtained: PPP, upper layer; CGF, middle layer; RBC, lower layer (A). CGF was removed from the tube, using sterile tweezers and cut the red part (RP) at 0,5 cm under buffy coat (BC) (B). The obtained CGF consists in three parts: the upper white part (WP), the downer red part (RP) and the middle BC part (C). Bar = 1 cm.

Diagnostic BioSystems, Pleasanton, CA). All sections were processed using UltraVision Quanto Detection System Horseradish Peroxidase (HRP; ThermoScientific, Bio-Optica, Milan, Italy), followed by development with diaminobenzidine (Amresco, Prodotti Gianni, Milan, Italy). Finally, they were counterstained with hematoxylin, dehydrated and mounted. The immunohistochemical control was performed by omitting the primary antibody, in presence of isotype-matched IgGs and performing pre-adsorption assay using the related peptide and gave negative results.

Platelet count

The indirect analysis of cellular component of CGF was performed on liquid re-mixed blood samples obtained after centrifugation with the same program to obtain CGF, using sterile Vacuette tubes with heparin (Greiner Bio-One, GmbH, Kremsmunster, Austria) to avoid coagulation. The hematologic blood test (leukocytes formula, platelets and erythrocytes) was performed by laboratory S.T.E.M. analysis (Brescia, Italy).

Scanning Electron Microscopy (SEM) Analysis

The samples of the CGF layers were fixed in 2% glutaraldehyde for 1 hour and then they were rinsed in cacodylate buffer solution and fixed for 1 hour with 1% osmium tetraoxide (OsO_4). Subsequently, the samples were dehydrated serially in 30, 50, 70, 90, and 100% ethanol solutions. The SEM procedures were completed by critical drying point of the material. Finally the CGF samples were observed at 20 kV using SEM LEO 1430 scanning electron microscope.

Cumulative growth factor release

The kinetics of PDGF-AB, VEGF, TNF-α, TGF-β1, IGF-I, BDNF and BMP-2 released from CGF clots were evaluated in triplicate by incubation of the CGF with RPMI 1640 cell medium (Lonza, Verviers, Belgium) without growth supplements for 5 hours, 1, 3, 6, 7 and 8 days (34). The CGF were placed in a 12-well plates (one in each well) with the addition of 1.6 ml of cell culture medium and then incubated at 37°C. After each incubation period, the medium was collected and centrifuged at 400 g for 10 min at room temperature. The supernatant was stored at −80°C until analysis [36]. The quantification of growth factors was performed using ELISA kits according to the manufacturer's protocol (R&D Systems Inc, Minneapolis, Minnesota, USA). The total quantity of growth factors present in the medium recovered at all time points was checked and reported both as mean value of all volunteers and as mean value of each volunteer at each time point.

In vitro cell proliferation

The CGF was prepared at the right time point of the experiment. Each CGF was placed into a sterile transwell insert (ThinCert™ cell culture inserts, Greiner Bio-One, Austria) with a semi-permeable membrane at the bottom and inserted into the 6-well plates (an insert in each well) for 72 hours. At the end of each treatment, the *in vitro* effect of CGF on cell proliferation was evaluated. Each experiment was performed in triplicate to ensure reproducibility of results and also to ensure a sufficient cell number for FACS analysis. At the end of the experiments, cell proliferation and morphology were evaluated in the three different cell lines used.

NHDF (Normal human dermal fibroblasts)

NHDF (cell derived from skin of adult donor; Lonza, USA) were cultured in Fibroblast Growth Medium (FGM; Lonza, Walkersville MD, USA) constituting by Fibroblast Basal medium (FBM; Lonza, Walkersville MD, USA) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (rhFGF-B, insulin, fetal bovine serum - all from BulletKits®, Lonza, Walkersville MD, USA), at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence. The medium was changed every 2 days. NHDF from the third and sixth passage were used in the experiments. At confluence, NHDF were passaged and seeded, at a final density of 10000cell/cm^2, in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in FBM for 24 hours, before stimulation. The medium was then removed and four different treatments were tested for 72 hours: 1) only FBM, 2) only FGM, 3) FBM with whole solid CGF and 4) FGM with whole

solid CGF.

HUVEC (Human Umbilical Vein Endothelial Cells)

HUVEC (pooled cells; Lonza, USA) were cultured in Endothelial Growth Medium (EGM; Lonza, Walkersville MD, USA) which consisted of Endothelial Basal Medium 2 (EBM2; Lonza, Walkersville MD, USA) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (hFGF, VEGF, IGF-1, hEGF, fetal bovine serum - all from EGM-2 Single Quot®; Lonza, Walkersville MD, USA) at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, with the medium changed every 2 days. Experiments were performed using HUVEC between the third and sixth passage. At confluence, HUVEC were passaged and seeded, at a final density of 10000cell/cm², in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in EBM2 for 24 hours, before stimulation. Subsequently, the medium was removed and four different treatments were tested for 72 hours: 1) only EBM2, 2) only EGM, 3) EBM2 with whole solid CGF and 4) EGM with whole solid CGF.

HOB (Human Osteoblasts)

HOB (cryopreserved cells; Promocell, Germany), were cultured in Osteoblast Growth Medium (OGM; Promocell, Heidelberg, Germany) which consisted of Osteoblast Basal Medium (OBM; Promocell, Heidelberg, Germany) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and SupplementMix (OGM Supplement Mix; Promocell, Heidelberg, Germany) containing growth factors (not specified by the manufacturer) at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence. The medium was changed every 2 days. Experiments were performed using cells between third and sixth passage. At confluence, HOB were passaged and seeded, at a final density of 5000cell/cm², in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in OBM for 24 hours, before stimulation. The medium was then removed and four different treatments were tested for 72 hours: 1) only OBM, 2) only OGM, 3) OBM with whole solid CGF and 4) OBM with whole solid CGF.

FACS analysis

Cells were detached with the Trypsin (0.025%)/EDTA (0.01%) solution (Promocell, Heidelberg, Germany) and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant, pellet was re-suspended in the appropriate culture medium. Cell suspension (100-200 μl), was transferred into each fresh tube (100000cells/tube) and permeabilized with Saponin (1 ml/tube), on ice for 10 minutes, preserving Ki-67 antigen. At the end of the incubation period with Saponin, cells were centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. Cells were stained with the mouse monoclonal antibody Ki-67 FITC-conjugated (BD Bioscience, San Diego, CA). 20 μl of Ki-67 antibody were added to each tube and incubated in the dark for 30 minutes, at 4°C. As a positive control the Ki-67 isotype control (BD Bioscience, San Diego, CA) was used and as negative control the primary antibody was omitted and only a secondary FITC antibody was used. Cells were then washed with FACS buffer (PBS with 2% FBS- 2 ml/tube), centrifuged at 1200 rpm for 5 minutes and re-suspended in 0.5ml of FACS buffer (PBS with 2% FBS). Finally, the cell samples were analyzed with FACS (BD FACSCanto™- BD Bioscience, San Jose, CA) and the data were analyzed using the BD FACSDiva™ software version 8.8.7 (BD Bioscience, San Jose, CA).

Mechanical characterization

Compression tests were carried out on three CGFs, one for each donor. Each CGF, after being removed from the glass tube used for centrifugation, was cut with a steel scalpel into two pseudo-cylindrical specimens: one obtained from the white part and the other obtained from the buffy coat part. For each specimen, the initial height (h_0) and cross-section (A_0), were indirectly measured on the photograph of each specimen, captured with a high magnification photo-camera (Nikon D7000). The software ImageJ (v. 1.47) was used for the image analysis. A_0 values were typically between 100 and 150 mm² and h_0 values between 3 and 6 mm. The compression tests were performed at room temperature, using an Instron test system (model 3366) equipped with a 50 N load cell. A crosshead speed of 2 mm/min was used. To prevent the samples drying, they were wetted with some drops of saline solution (0.9% NaCl), before the beginning of each test.

For each specimen, starting from the load vs crosshead displacement curve, the nominal stress (σ) vs nominal strain (ε) curve was constructed. The nominal strain (ε) was evaluated as:

$$\varepsilon = 1 - \frac{h}{h_0} \qquad (1)$$

where h and h_0 are the actual height (measured directly from the crosshead displacement) and the initial height of the specimen, respectively. The nominal stress (σ) was evaluated as:

$$\sigma = \frac{F}{A_0} \qquad (2)$$

where F is the recorded load and A_0 the initial cross-section of the specimen. A reference test, carried out without the specimen in place, was also performed in order to verify the value of h_0, evaluated from the photographic analysis.

Statistical Analysis

One-way ANOVA test corrected by Bonferroni was used for statistical analysis. A P-value less than 0.05 was considered statistically significant. Results were expressed as mean ± standard error (SE).

Results

Blood analysis

The blood analysis laboratory confirmed that the tested subjects presented normal blood values and could be included in the experimental protocols.

Morphological characterization

Blood cells: The fibrin matrix appeared homogeneous in a light brown colour in MGG (Figure 2A) and a light pink colour in HE (Figure 2B). The leukocyte nuclei were stained in dark blue in MGG (Figure 2A) and violet in HE (Figure 2B). The erythrocytes were stained in light brown in MGG (Figure 2A) and pink/red in HE (Figure 2B). So, leukocytes and erythrocytes were clearly detected by both MGG and HE staining. The leucocytes were localized principally in the buffy coat but also scattered in the white and red part of CGF, close to buffy coat. The erythrocytes were present only in the red part of CGF. The platelets, because of their small size, were difficult to be visualized using these classic stains in a context where the fibrin network created a troubled background.

The immunohistochemistry for CD61, a platelet marker, showed a clear positivity in the buffy coat of CGF in all specimens analysed (Figure 2C). These data were supported by SEM analysis (Figure 2C inset). Platelets were also scattered in the white part of CGF, where
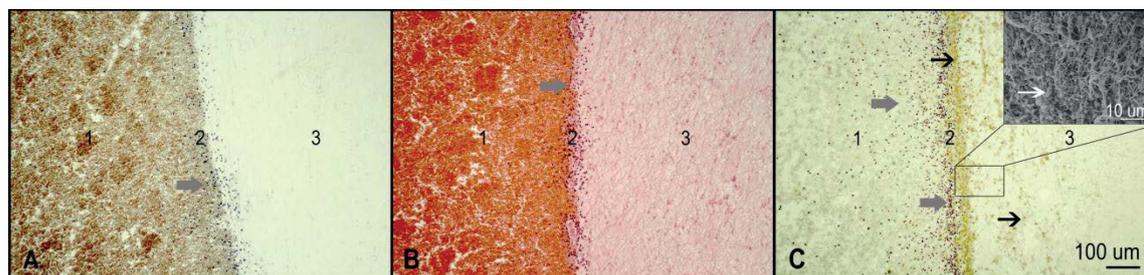
Figure 2: Micrographs of CGF representing (1) red part, (2) buffy coat, (3) white part. A) HE staining, B) MGG staining, C) CD-61 immunostaining for platelets; the inset represent platelets in the buffy coat obtained by SEM analysis. The black arrows (white in inset) indicate platelets, the gray arrows indicate leukocytes.
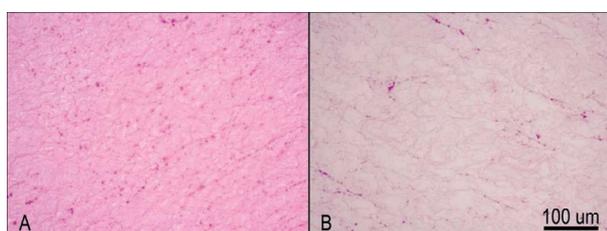
Figure 3: Micrographs of CGF fibrin network in white part (A) near the buffy coat and (B) far from the buffy coat stained with HE.

formed aggregates, trapped in the fibrin network.

Fibrin network: Images of the CGF fractions by close to and far from the buffy coat were clearly detected by light microscopy, using EE staining (Figure 3). The images showed that the fibrin network and architecture changed moving from the buffy coat to the white part. In particular, close to the buffy coat, the fibrin network was strictly compact (Figure 3A) while those far from the buffy coat became a larger mesh (Figure 3B).

Platelet count: The blood subjected to CGF centrifugation program had a loss of platelets but the number of erythrocytes and leukocytes did not change. The recovered platelets represented the 50.04% ± 7.62. Therefore, we could suppose that the missing platelets were destroyed during CGF centrifugation.

Evaluation of cumulative growth factor release

The results varied greatly among volunteers.

Considering the mean value of the three volunteers, we can say that:

- PDGF-AB has a constant accumulation during the experimental time (Figure 4a),

- VEGF increases during all the experimental period, reaching the maximum accumulation at 8th day (Figure 4b),

- TNF-α reaches the maximum accumulation at the 1st day and then progressively decreases until the 8th day (Figure 4c),

- TGF-ß1 has a constant accumulation during all the experimental period (Figure 4d),

- BDNF reaches the maximum accumulation at the 1st day and after this it maintains a plateau during the experimental time (Figure 4e),

- BMP-2 reaches the maximum accumulation at 8th day (Figure 4f), so its release is maximum in the last part of the experimental period,
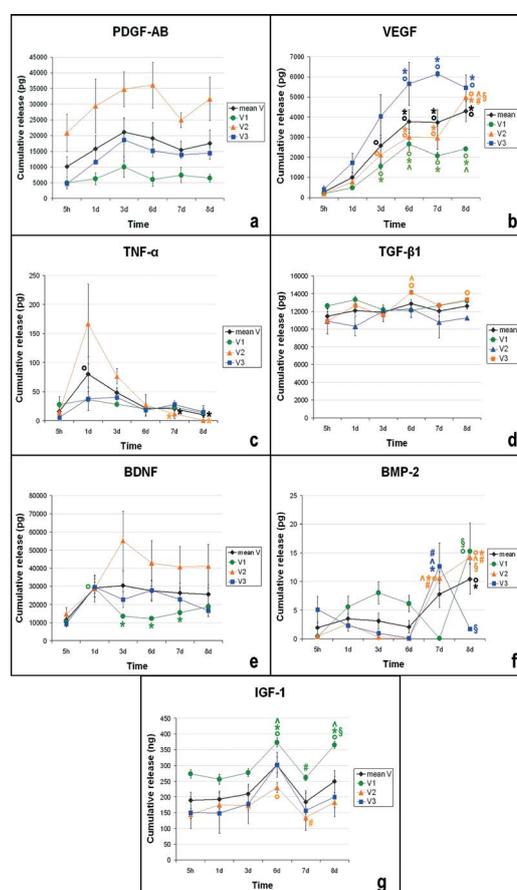

Figure 4: Cumulative growth factor released by CGF at 5 hours (5h), 1 day (1d), 3 days (3d), 6 days (6d), 7 days (7d) and 8 days (8d) of culture. Each volunteer's values are plotted (V1,V2,V3) as well as their mean value (mean V).The values are reported as total amount of growth factor released ± SE. °$P<0.05$ vs 5h; *$P<0.05$ vs 1d; ^$P<0.05$ vs 3d; #$P<0.05$ vs 6d; §$P<0.05$ vs 7d. (a) PDGF-AB, (b) VEGF, (c) TNF-α, (d) TGF-β1, (e) BDNF, (f) BMP-2 and (g) IGF-1.

- IGF-1 reaches the maximum accumulation at 6th day and then it decreases (Figure 4g).

### Cell proliferation assay and morphology

The proliferative effect of CGF on NHDF, HUVEC and HOB was assessed using FACS analysis, by measuring the expression of intracellular proliferation marker Ki-67. The results are described in detail below.
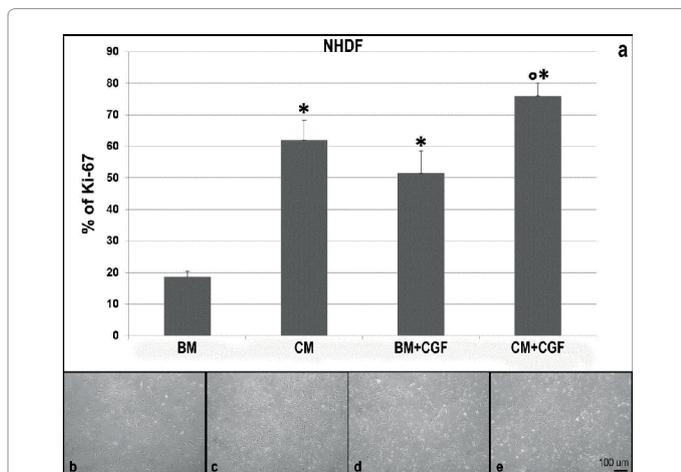
Figure 5: Effect of CGF on NHDF. (a) Ki-67 expression in NHDF grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0.05 vs BM+CGF; ^P<0.05 vs CM.(b-e) Micrographs of NHDF after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.
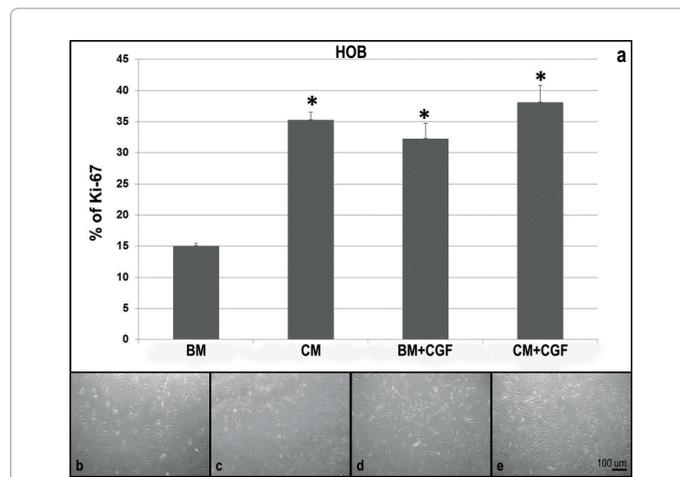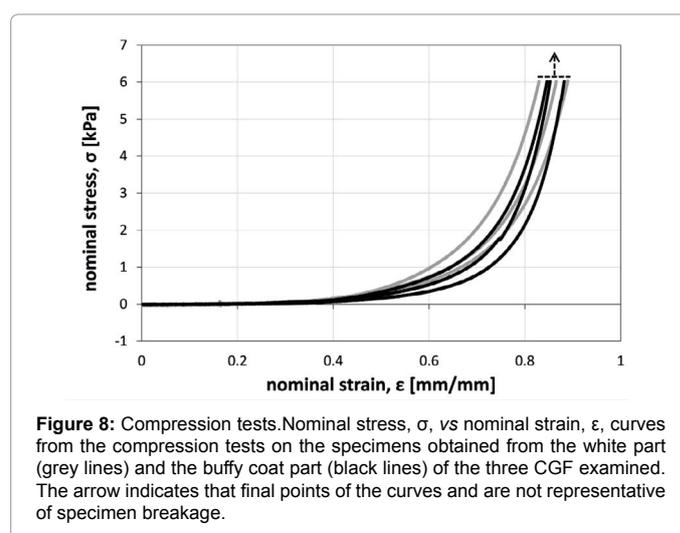

Figure 7: Effect of CGF on HOB. (a) Ki-67 expression in HOB grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0.05 vs BM+CGF; ^P<0.05 vs CM.(b-e) Micrographs of HOB after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.
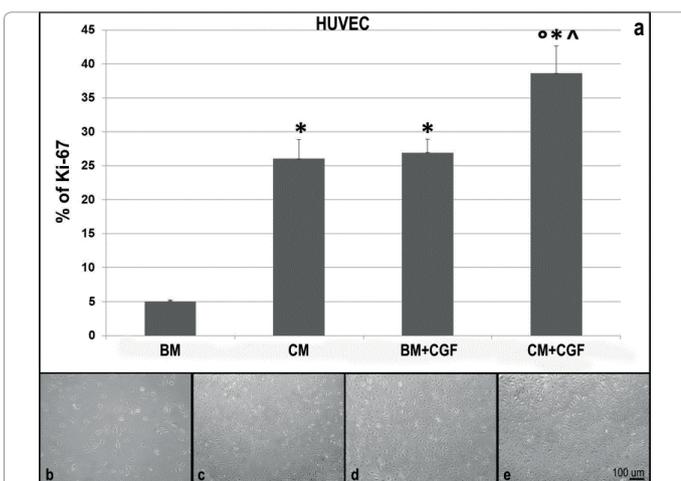
Figure 6: Effect of CGF on HUVEC. (a) Ki-67 expression in HUVEC grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0,05 vs BM+CGF; ^P<0.05 vs CM. (b-e) Micrographs of Huvec after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.

Figure 8: Compression tests.Nominal stress, σ, *vs* nominal strain, ε, curves from the compression tests on the specimens obtained from the white part (grey lines) and the buffy coat part (black lines) of the three CGF examined. The arrow indicates that final points of the curves and are not representative of specimen breakage.

	M_{small} [kPa]	M_{large} [kPa]
white part	0.70 ± 0.23	41 ± 1.9
buffy coat part	0.45 ± 0.13	54 ± 6.8

Table 1: Average values of the moduli M_{small} and M_{large}, evaluated at small and large deformations, respectively, for the white and the buffy coat parts of the CGF samples.

NHDF

In basal medium, free of serum and growth factors, the cells showed a low percentage of Ki-67 positivity (18.68% ± 1.8) (Figure 5a). The cells did not show a clear spindle-like morphology, appearing short and not well spread over the plate surface (Figure 5b). In the complete medium, the percentage of Ki-67 positive cells increased (61.98% ± 6.35) compared with the basal medium (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing elongated and well spread over the plate surface (Figure 5c). In the basal medium with CGF, the percentage of Ki-67 positive cells also increased (51.51% ± 7.12) (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing elongated but not well spread over the plate surface (Figure 5d). In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (75.9% ± 4.13) compared with basal medium alone and with CGF (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing well elongated, larger and well spread over the plate surface compared with all the other culture conditions (Figure 5).

HUVEC

In the basal medium, free of serum and growth factors, the cells showed a very low percentage of Ki-67 (5.05% ± 0.19) positivity, being cell growth very influenced by culture conditions (Figure 6a). The cells showed a round shape morphology and appeared not well attached over the plate surface (Figure 6b). In the complete medium, the percentage

of Ki-67 positive cells markedly increased (26.03% ± 2.79) compared with the basal medium (Figure 6a). The cells showed the typical polygonal shape morphology appearing well attached over the plate surface (Figure 6c).

In the basal medium with CGF, the percentage of Ki-67 positive cells markedly increased (26.94% ± 1.96) (Figure 6a). The cells showed a more defined polygonal shape morphology appearing larger and well attached over the plate surface compared with basal and complete medium alone (Figure 6d).

In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (38.62% ± 4.03) compared with all the other culture conditions (Figure 6a). The cells showed a more defined polygonal shape morphology appearing larger and well attached over the plate surface, compared with all the other culture conditions (Figure 6e).

HOB

In the basal medium, free of serum and growth factors, the cells showed a low percentage of Ki-67 positivity (15.07% ± 0.39) (Figure 7a). The cells showed their typical polygonal and flattened shape morphology and appeared well attached over the plate surface (Figure 7b). In the complete medium the percentage of Ki-67 positive cells markedly increased (35.31% ± 1.21). compared with the basal medium (Figure 7a). The cells showed a more elongated polygonal and flattened shape morphology with the presence of extensions or very thin filopodia compared with the basal medium (Figure 7c). In the basal medium with CGF, the percentage of Ki-67 positive cells markedly increased (32.3% ± 2.46) compared with the basal medium alone (Figure 7a). The cells showed a more elongated, larger polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia compared with the basal medium and complete medium (Figure 7d). In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (38.13% ± 2.72) compared with all the other culture conditions (Figure 7a). The cells had a more elongated polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia (Figure 7e).

Mechanical test

The nominal stress (σ) vs nominal strain (ε) curves obtained from the compression tests, are reported in Figure 8. Each test was interrupted at a large strain level, close to 0.9 mm/mm, and, once the load was removed, the specimen did not recover the deformation they had undergone during the compression test, but remained largely deformed. The mechanical response of the material appeared only slightly dependent on the part of the CGF examined (whether the white or the buffy coat part). Irrespective of the CGF part considered, the material exhibited a non-linear nominal σ-ε behavior, and became apparently stiffer as the strain level increases. From the nominal σ-ε curve of each specimen, two characteristic moduli, M_{small} and M_{large}, representative of the material stiffness at small and large deformations, respectively, were evaluated. M_{small} was evaluated as the slope of the line secant to the σ-ε curve at two fixed levels of strain (0.2 and 0.4 mm/mm) in the small strain region, whereas M_{large} as the slope of the secant at two fixed levels of stress (4 and 5 kPa) in the large strain region of the curve (at ε generally higher than 0.8 mm/mm). For each of the two parts of the CGF sample examined (white and buffy coat, respectively), average values of M_{small} and M_{large} were evaluated from the data obtained for the three different samples, and they are reported in Table 1. The results showed that, at small strains, the material from the white part appears, on average, to be slightly stiffer than that from the buffy coat,

whereas, at large strains, the buffy coat part appeared to be the stiffest.

Discussion

The first aim of this study was to evaluate the blood cell localization in CGF. Our results have shown that platelets are localized in the buffy coat, in a very thin space. Considering the platelets localization, the volume of the white part of CGF could be reduced, if necessary, in clinical practice. In the literature, the morphology of PRF, at the light microscopy, was performed [14]. The blood cells were localized in the buffy coat using two stains (Hematoxylin/Eosin and Masson's trichrome modified by Goldner) but without obtaining a totally clear identification of the platelets. Moreover, an immunohistochemical localization of platelets in PRF membranes corroborated by a scanning electron microscopy analysis was also performed [19]. Platelets were concentrated on the surface of the region adjacent to the red thrombus and, considering two different types of compression, or either on one side of the surface or inside the membrane.

With regard to the quantity of recovered platelets, it is important to consider that, in our experiments, about the half of them are destroyed during centrifugation. This data support the hypothesis that CGF guarantees an immediate available free quantity of growth factors. In PRF [14], the count of blood cells was done on the clot exudates after compression. There was a decrease of platelets and leucocytes of approximately 97% and >50% respectively. In other liquid PRP preparations, the count of blood cells was easily performed using a routine haematology analyser. Some authors, using newly optimized PRP preparation method, obtained a final platelet recovery of 46.9 to 69.5% [37]. Bertrand-Duchesne et al. [38], using a platelet concentrate collection system (PCCS), reported a platelet recovery rate of 63% (range, 44.1-75.0%); Leitner et al. [39] compared different PRP systems with a collection efficiency ranging from about 13 and 78%. All these works support our data, nevertheless other articles report the number of recovered platelet as concentrations (number of platelets/volume) and usually do not mention any possible loss of cells and so cannot easily be compared with our data [40-44].

The second aim of this study was to evaluate the *in vitro* cumulative release of growth factors. Our data show that each growth factor has a specific kinetic of accumulation, probably due to a different accumulated quantity in α-granules and a different accumulation of mRNA in platelets useful to synthesize growth factors up to over 7 days after activation [45]. The different kinetics suggests a precise and programmed release to sustain regeneration. Furthermore, the growth factor concentrations varied greatly among the volunteers' samples, suggesting that the results could be influenced by the individual biological characteristics. In fact, Weibrich et al. [46], suggested that different individuals may require different platelet concentration ratios to achieve a comparable biological effect. Marx [2] did suggest however that $1×10^6$ platelet/μL should be set as the threshold concentration of therapeutic PRP in order to ensure a therapeutically effective amount of growth factors in PRPs.

We therefore decided to report the quantity of growth factors as an absolute value and not as concentration as it sometimes referred to in the literature. This decision was made considering the solid nature of CGF and the necessity to clearly understand in clinical practice the real amount of bioactive molecules at disposable for patients at a specific time point.

PDGF and TGF-β1 are the majority growth factors and the most important in PRPs and many reports focus especially on PDGF, TGF-β1

and IGF [47-50]. In our experiment we observed a mean constant accumulation of PDGF-AB and TGF- β1. PDGF-AB is involved in all three phases of wound healing, including angiogenesis, the formation of fibrous tissue and re-epithelialization [51]. Our results suggest a clear constant PDGF-AB release, with a mean range between 10000 and 20000 pg, assuring a steady contribution to wound healing repair. A similar kinetic was found for TGF- β1, a multi-functional cytokine. In our experiment, TGF- β1 has a mean value of about 12000 pg. even if it seems that the ideal PRP concentration is when the TGF-β1 is between 50 and 100 ng/ml [42]. Furthermore, in a rat tibial fracture model, injections of TGF-β (4 and 40 ng) every other day for 40 days caused a dose-dependent increase in bone thickness. As regards IGF-1, it reaches the maximum accumulation at 6th day and then it decreases. IGF-1 has an anabolic effects and in our experiment, it has a mean value of around 200-300ng. This data is supported by another study where the content in IGF-1 remained close to 250 ng along the time course of the study. This agrees with other previous observations that IGF-1 is in majority present in plasma [52,53] and therefore was not expected to increase as a consequence of platelet activation and release from PRF [54].

On the other hand, VEGF and BMP-2 have only a slow kinetic release. VEGF, which increases angiogenesis and vascular permeability [55], has a linear kinetic release, reaching the maximum accumulation and so therefore its plateau, at the end of the experimental period with about 4000 pg. BMP-2, which induces osteoblast differentiation with osteo-inductive properties, has a slow kinetic with a linear increase from the 6th day and so reaching the maximum accumulation at the end of the experimental period with only about 10 pg. Only a few studies have analysed the contribution of BMP-2 from PRP during the healing process. One of these evaluated BMP-2 quantity in PRP which was the lowest compared with the other growth factors analysed [56]. The other one demonstrated that PRP accelerated bone fracture healing of rat femurs via modulation of BMP-2 and also TGF-β1 and growth factor expression [57].

Nevertheless, TNF-α and BDNF have a fast kinetic release, considering the mean value. TNF-α reaches the maximum accumulation at 1 day and after this decreases. It has controversial effects and elicits a variety of responses, depending on the cellular context [58]. In fact, it is able also to induce inflammatory response as it is also produced by leukocytes. TNF-α is also present in PRP [37] and in activated PRP. Bendinelli et al. [59] observed increases in TNF-α, hepatocyte growth factor (HGF) and IL-4. TNF- α and HGF, by disrupting NF-kB-transactivating activity, are important for the anti-inflammatory function in this context. BDNF is known to be an important member of the neurotrophic family with neural growth and differentiation properties and the large amounts of circulating BDNF proteins are stored in platelets [60,61]. It reaches the maximum accumulation at 1day (about 30000 pg) and after this maintains a plateau. This is a growth factor that is not usually investigated in PRPs but, considering some interesting results on its application in neurodegenerative pathologies, such as Alzheimer's disease [62], we have included it in our investigation.

In a recent study [54], PDGF-AB, TGF-β1 and VEGF content released from PRF releasate increased dramatically and gradually over the time course of the study. Content in the releasate reached mean values of 52.37, 72.21, 1.04 ng at 300 minutes for PDGF-AB, TGF-β1, EGF, and VEGF respectively. The values were greater compared with ours but this was probably due to different methods of evaluation. By contrast, the IGF-1 content remained stable at about 250 ng, as in our experiment. Another study on growth factor release was performed by

Anitua et al. [36] showing that the growth factor delivery is diffusion controlled with a rapid initial release by 30% of the bioactive content and a steady state release when almost 70% of the growth factor content is delivered. In particular, the kinetics of PDGF-AB and VEGF release from PRGF clot is linear reaching a plateau after the 3rd experimental day. In our experiments, we did not observe a linear accumulation in all the growth factors analyzed probably due to short half-life, proteolitic processes and decrease of growth factor release [63-65]. In particular, PDGF-AB was linear up to 3rd day, while VEGF was linear up to 6th day when it reached its plateau. These data are supported by a recent paper where the growth factor release of L-PRF from 8 hours to 28 days was evaluated, showing a non-linear kinetic release [47]. As shown above, there are different results in the literature concerning the quantitative measurement of the growth factors contained in PRPs [48,49]. Such differences seem to be multifactorial and include inter-patient variability of the proteins amount contained in the platelets, different degrees of platelets concentration during PRP preparation, activation or inactivation, as well as different degrees of platelet membrane breakage and the specific degree of platelet activation at the time of measurement [66].

The third aim of our study was to assess the *in vitro* CGF growth potentiality, on three different human cell lines: NHDF, HUVEC and HOB. The activity of growth factors released by CGF clearly influences the cell growth.

The proliferative effect of CGF was evident in all three cell lines. However the mitogenic potential of CGF was more evident when added to the basal medium, especially in HUVEC and HOB cells, where the percentage of Ki-67 positive cells was similar with both the basal and complete medium. Moreover, except than in fibroblasts, CGF addition to complete medium, did not enhance the growth effect of medium standard supplements, most probably because the cells were already fully stimulated.

NHDF grow in presence of bFGF (basic Fibroblast Growth Factor), IGF-1, EGF (Epidermal Growth Factor), PDGF and TGF-β. Some data are particularly interesting regarding the role of specific growth factors on NHDF proliferation. Indeed the results of Gasparri et al. [67] and Okuda et al. [68] confirm that PDGF, bFGF and TGF-β potently induce NHDF to proliferate, as previously reported [69,70]. On the other hand, EGF and IGF-1 also stimulate proliferation [71,72] but less potently than PDGF or bFGF. Furthermore, consistent with our results, a recent work [73] reports that PRP markedly increases fibroblast proliferation compared with the serum-treated control group.

Also in HUVEC, the CGF supplementation positively influences cell growth, but less so than in fibroblasts. HUVEC proliferate in presence of specific growth factors such as EGF, VEGF, bFGF and IGF-1. Indeed it has been shown that these growth factors are, at least in part, responsible of the PRP [38] and PRGF [74] mitogenic and angiogenetic effect for endothelial cells.

As regards HOB, here CGF was able to significantly stimulate their *in vitro* proliferation. The mechanism responsible for the cell proliferation by CGF may be explained as follows: CGF is rich in a variety of growth factors, such as TGF-β, PDGF-AB, VEGF and IGF-I [18]. It has been demonstrated [75] that these growth factors seem to be highly relevant for osteoblast proliferation and differentiation, suggesting a beneficial effect of platelet preparations on HOB growth and viability [76]. In particular, PDGF and TGF-β1 seem to be the most dominant factors which significantly contribute to HOB proliferation [77]. As regards IGF-1, Ogino et al. [77] suggest that this growth factor is

able to enhance osteoblast proliferation when it is combined with other growth factors such as PDGF and TGF-β1. Moreover, unlike PRP, CGF does not dissolve rapidly after application, instead, the strong fibrin gel in the matrix addition is slowly remodeled in a similar manner to a natural blood clot. Thus, CGF prolongs the duration of growth factor action and enhances cell proliferation and osteogenic differentiation.

Furthermore, due to its higher strength and viscosity, CGF could better protect the growth factors from proteolysis, compared with PRP and PRF [63]. This seems to agree with our *in vitro* results, where the CGF addition enhances cell proliferation in all the three different cell lines (fibroblasts, endothelial cells and osteoblasts) involved in angiogenesis, tissue remodeling and regeneration. The results concerning other PRP preparations support this data. The capacity of two human blood fractions (the supernatant serum and the releasate PRF) were evaluated monitoring the cell proliferation in two human cell lines, human embryonic kidney fibroblasts HEK293 and human MG-63 osteoblastic cell line. The supernatant serum and the releasate PRF at 1-10%, stimulated cell growth more significantly than FBS-free medium [78]. It is interesting that the addition of platelet lysate-plasma, obtained from pooled PRP, to human mesenchymal stromal cell (hMSCs) culture showed a consistently greater proliferation rate than with FBS at the same percentage [79].

The fourth aim of the study was to analyze the mechanical behaviour of CGF. Specimens did not recover from deformation once the compression load was removed. This "apparent plasticity" seems to indicate that, under compression at high strain levels (similar to the deformational state experienced by CGF during positioning in the patient), the material microstructure undergoes modifications that can have permanent character. Regarding its non-linear nominal σ-ε behavior, it is interesting to note that literature works report that a strain-stiffening effect is observed for fibrin gels under shear deformation [80]. For the CGF materials here examined, the volume variations which the specimen underwent during the compression test did not allow the construction of reliable true stress vs true strain curves. Consequently, at this stage of the work, it is not possible to confirm that the apparent strain-stiffening effect observed here is intrinsic to the material. Further experiments are needed to better investigate this aspect.

In relation to the moduli M_{small} and M_{large}, it can be reasonably thought that, at large strains, the mechanical response of the material under analysis is governed by the architecture of the fibrin network. With this in mind, the higher M_{large} exhibited by the buffy coat part would reflect the tighter structure of the fibrin network observed in this zone of the CGF sample by histomorphological analysis, compared to that observed in the white part.

It seems clear that CGF represents a plasma preparation with great potentiality, whose clinical use seems to be promising. Indeed, our data underline that the growth factors present in CGF influence in a specific way the different cell types. CGF has a solid-like nature, it is high compliant, can be easily handled and could be useful for specialized application in regenerative medicine, dentistry, oral implantology and cell therapy.

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