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RESEARCH ARTICLE

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How the different material and shape of the blood collection tube influences the Concentrated Growth Factors production

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Abstract

Platelet concentrates, such as Concentrated Growth Factors (CGF), are autologous preparations obtained from the patient's own blood and rich in platelets, growth factors and cytokines involved in the key processes of tissue regeneration. These autologous concentrates differ in the way of preparation and also in the content of platelets, growth factors and leucocytes, as well as in the fibrin network architecture. So it is difficult to have a standardized product. The aim of the present study was to evaluate how the use of test tubes of different material, for blood collection, could influence the CGF production. Three different test tubes were used and the obtained CGFs were subjected to histomorphological and immunohistochemical analyses. Results showed that the tube material and shape influenced the CGF composition. In fact, according to the type of tube used, the obtained CGFs showed differences in morphology, in the fibrin network architecture and in blood cell localization and distribution.

KEYWORDS

Blood, CGF, glass and plastic blood collection tubes, fibrin architecture, leukocytes, platelets

1 | INTRODUCTION

Platelet concentrates are autologous preparations (Mehta & Watson, 2008; Prakash & Thakur, 2011) obtained from the patient's own blood, by a specific protocol of centrifugation. These preparations contain autologous platelets, several different growth factors (such as PDGF, TGF-β1, VEGF, IGF-1, TNF-α) as well as other cytokines involved in the key processes of tissue regeneration including cell proliferation and differentiation, extracellular matrix synthesis, chemotaxis, and angiogenesis (Anitua et al., 2015; Martínez, Smith, & Palma Alvarado, 2015; Schär, Diaz-Romero, Kohl, Zumstein, & Nesic, 2015). Growth factors and cytokines are contained in platelet secretory granules and they are released from activated platelets, so promoting healing in both soft and hard tissues (Agarwal, Agarwal, & Kumar, 2013; Naik, Karunakar, Jayadev, & Marshal, 2013; Oryan, Alidadi, & Moshiri, 2016).

Actually, different blood preparations have been developed, which differ for the leukocyte and platelet content, the fibrin architecture, and the protocol of preparation. So it is difficult to have a standardized product. In fact, leukocyte content and fibrin architecture are two key characteristics of all platelet concentrates which allow to classify these technologies in five main categories (Dohan Ehrenfest, Rasmusson & Albrektsson, 2009; Rodella & Bonazza, 2015): (1) Pure Platelet-Rich Plasma (P-PRP), such as cell separator PRP, Vivostat platelet rich fibrin (PRF), or Anitua's PRGF; (2) Leukocyte and Platelet-Rich Plasma; (3) Pure Plaletet-Rich Fibrin, such as Fibrinet; (4) Leukocyte- and Platelet-Rich Fibrin, such as Choukroun's PRF, and (5) Concentrated Growth Factors (CGF).

CGF is obtained through a specific and standardized protocol of centrifugation which requires a special programmed centrifuge (Medifuge MF200, Silfradent srl, Forli, Italy) and specific plastic tubes, coated with silica microparticles (Greiner Bio-One, GmbH, Kremsmunster, Austria), for blood collection, without the addition of exogenous substances (Borsani et al., 2015; Chen et al., 2016; Rodella et al., 2011). This method allows to separate CGF in three different fractions: the upper white part (WP), the downer red part (RP), and the middle "buffy coat" (BC), interface between white and red part. CGF has a gelatinous consistency with platelets localized in BC and releases growth factors for almost 8 days (Borsani et al., 2015). In particular, it contains autologous osteo-inductive growth factors derived from platelets and an osteo-conductive fibrin matrix (Honda, Tamai, Naka, Yoshikawa, & Myoui, 2013). Up to now it has been used especially in dentistry and

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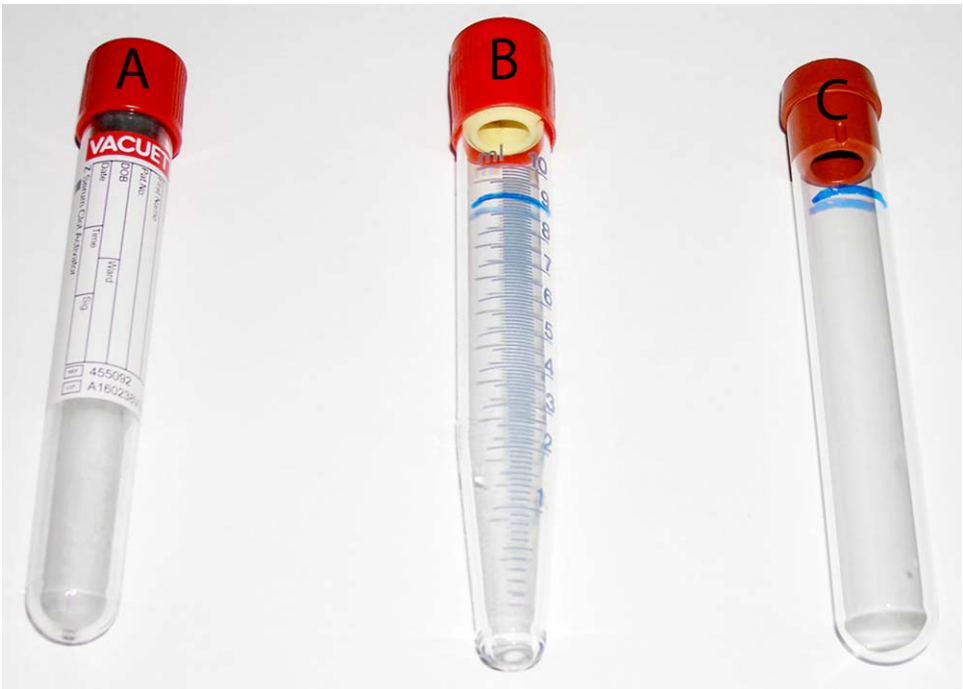

FIGURE 1 Different test tubes used for blood collection: (a) plastic silica coated tube, (b) glass conical bottom tube, (c) glass round bottom tube. [Color figure can be viewed at wileyonlinelibrary.com.]

maxillofacial surgery (Bettega et al., 2009; Sohn et al., 2011) but also *in vitro* to enhance the migration and proliferation of human dental stem cells and gingival fibroblasts (Yu & Wang, 2014).

The aim of the present work was to evaluate how the use of different tubes for blood collection could influence the CGF production.

2 | MATERIALS AND METHODS

2.1 | Blood collection

For the experiments, the same amount of venous blood was collected by piercing a superficial vein with a 21-gauge needle, from three healthy adult volunteers (V1, V2, and V3) of Caucasian ethnicity, with red blood cells, platelets and leukocytes levels within the normal range and who have expressed their informed consent. Moreover, the exclusion criteria included: systemic disorders, smoking, infections, non-steroidal anti-inflammatory drug use and hemoglobin level <11 g/dL for females and <13.5 g/dL for males.

2.2 | CGF preparation

Three different protocols were used for each volunteer (V1, V2, and V3) to produce the CGF, varying for each one the blood collection tube composition and morphology:

- PROTOCOL 1: sterile Vacuette tubes (Greiner Bio-One, GmbH, Kremsmunster, Austria) coated with silica microparticles (SCTs) as a serum clot activator were used for blood collection (Borsani et al., 2015; Chen et al., 2016; Rodella et al., 2011) (Figure 1a).
- PROTOCOL 2: glass tubes with a round bottom (GRBTs) (Silfradent srl, Forli, Italy) were used for blood collection (Figure 1b)
- PROTOCOL 3: glass tubes with a conical bottom (GCBTs) (Silfradent srl, Forli, Italy) were used for blood collection (Figure 1c)

The different tubes were then immediately placed into the centrifuge (Medifuge, Silfradent srl, Forlì, Italy) and the blood samples were centrifuged using a program with the following characteristics: 30 sec acceleration, 2 min at 2,700 rpm, 4 min at 2,400 rpm, 4 min 2,700 rpm, 3 min at 3,000 rpm, and 36 sec deceleration and stopped. At the end of the centrifugation process, the CGFs were obtained.

2.3 | CGF analysis

The different CGFs were weighted and then processed and subjected to histomorphological and immunohistochemical analyses.

2.4 | Histomorphological analysis

Immediately after centrifugation, the different CGFs were collected and fixed in 10% neutral buffered formalin solution (pH 7.2) for 24 hr and embedded in paraffin according to standard procedures. Twenty serial sections (7 μm thick) of each sample were cut by a microtome, attached on microscope slides and subsequently used for the histomorphological and immunohistochemical analyses.

The morphology of CGFs was evaluated using the Hematoxylin and Eosin staining (HE; Bio-Optica, Milan, Italy), which is one of the principal stains in histology. According to the manufacturer's protocol, the paraffin sections were firstly deparaffinized and rehydrated. Then they were covered with Carazzi's Hematoxylin, for about 10 min, in the dark. After removing the excess dye with tap water, the sections were counterstained with Eosin for 1 min, dehydrated and mounted with

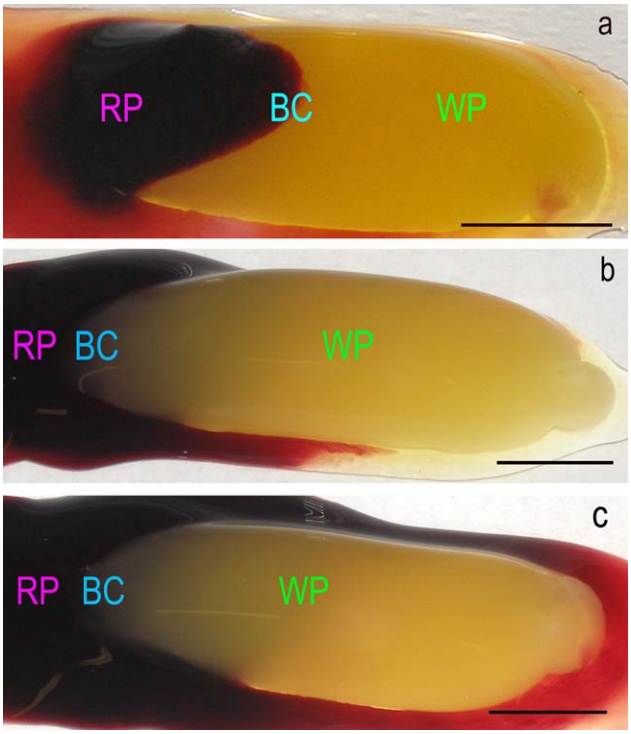

FIGURE 2 CGF obtained using: (a) plastic Silica coated tube, (b) glass round bottom tube, (c) glass conical bottom tube. Each CGF consists of three parts: the upper white part (WP), the downer red part (RP), and the middle buffy coat (BC). Bar = 1 cm. [Color figure can be viewed at wileyonlinelibrary.com.]

DPX (DPX Mountant for histology, Sigma Aldrich, Milan, Italy) for light microscopy detection.

For each CGF, we evaluated:

- the morphology, especially the BC and the WP;
- the fibrin network architecture, near and far from the BC;
- the blood cells localization and distribution

2.5 | Platelet immunohistochemistry

In order to visualize and localize platelets (not detectable using the HE staining), an immunohistochemistry with the platelet marker CD61 was performed for each CGF. According to the manufacturer's protocol, the sections of CGF in paraffin were firstly deparaffinized and rehydrated; then they were subjected to antigen retrieval in 0.05 M sodium citrate buffer (pH 6.0), in a water bath, set at 98°, for 20 min. After blocking endogenous peroxidase with a solution of 3% hydrogen peroxide (H_2O_2), the sections were incubated for 1 hr, at room temperature, with the mouse monoclonal antibody anti-human CD61 (platelet membrane glycoprotein IIIa, dilution 1:50, 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 the chromogen substrate Diaminobenzidine (DAB, Amresco, Prodotti Gianni, Milan, Italy). In order to better visualize the positive reaction, the sections were counterstained with Carazzi's Hematoxylin, dehydrated, and mounted with DPX, for light microscopy detection. CD61 negative control slides were obtained by omitting the primary antibody.

3 | RESULTS

After centrifugation, three blood fractions were identified in all the different tubes used: (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 of free red blood cells (RBC), and (3) the middle layer, representing the solid CGF, consisting of three fractions: the upper WP, the downer RP and the middle BC (Figure 2a–c).

3.1 | CGFs weights

The CGFs obtained using the PROTOCOL 1 with SCTs showed the lower weight (average weight of 2.589 g ± 0.38), compared with the CGFs obtained with the glass tubes, so used the PROTOCOL 2 and 3. In particular, the CGFs obtained with GCBTs (PROTOCOL 3) showed an average weight similar to the CGFs obtained using GRBTs (PROTOCOL 2), as reported in Table 1.

3.2 | CGFs morphological characterization

CGFs light microscopy analysis revealed that the samples showed a different morphology in relation to the type of tube used for blood collection.

3.3 | Buffy coat morphology

The CGFs obtained using the SCTs showed a clear and linear interface, which distinctly separates the red part from the white part (Figure 3a). Moreover, the erythrocytes were present only in the red part and not in the fibrin network. On the contrary, the other two types of CGFs, both those obtained with GRBTs and those obtained with GCBTs, did not show a linear interface and the erythrocytes were scattered also in the fibrin network, near the BC (Figure 3b,c).

3.4 | Fibrin network architecture

All the different types of CGFs showed variations also in the fibrin network architecture, moving from the interface to the white part. Fibrin network structure appeared very compact, with tight meshes in proximity to the BC and progressively became less compact, with large meshes far from it. CGFs produced using GCBTs showed the most compact fibrin network architecture, near the BC, compared with the other two types of CGFs. On the contrary, CGFs produced using the glass tubes showed a less compact fibrin structure in the WP, compared with CGFs obtained using SCTs.

3.5 | Platelet and leukocyte distribution

Immunohistochemistry with the platelet marker CD61 showed that platelet distribution varied according to the type of CGF analyzed. In

TABLE 1 Individual and average values of the weights of the different CGFs, evaluated for each volunteer

	V1 weight	V2 weight	V3 weight	Average of V1/V2/V3 ± SD
Si-coated tubes	3.116 g	2.461 g	2.192 g	2.589 g ± 0.38
Conical base glass tubes	3.421 g	3.372 g	4.182 g	3.658 g ± 0.37
Round base glass tubes	3.251 g	3.345 g	4.340 g	3.645 g ± 0.49

CGFs obtained with SCTs, platelets (brown color) were localized only in the BC, in a very thin space and leukocytes were present both in the RP and in the BC (blue nuclei), (Figure 4a).

In CGFs obtained with GRBTs, platelets were less present in the BC and more scattered in the WP (Figure 4b), while leukocytes appeared both in the RP and in the BC.

In CGFs obtained with GCBTs, platelets appeared scattered only in the WP while leukocytes were present also in the BC (Figure 4c).

4 | DISCUSSION

Our results showed that CGF varies according to the different composition and shape of the test tubes used for blood collection. The use of glass tubes seems to possess some advantages: first of all an easier manufacturing technology than plastic tubes. However, in glass tubes, blood begins to coagulate almost immediately after the collection and so the samples should be centrifuged very rapidly, in order to avoid a diffuse polymerization of fibrin, which is not ideal for tissue healing.

The first evidence is that the use of glass tubes allows to obtain larger CGFs that are longer, thicker and so with a higher weight compared with CGFs obtained using plastic silica coated tubes. This is probably due to an increase of amount of fibrin recruitment, so leading to a more extensive fibrin network and a greater plasma trapping. Moreover, both histomorphological and immunohistochemical analyses show that CGFs differ also in the fibrin network architecture, the BC morphology and the platelet and leukocyte distribution (Borsani et al., 2015; Rodella et al., 2011).

In addition, platelets and leukocytes are present not only in the BC but also in the WP. This could be advantageous when CGF is used as filler, so applying only the WP, fully discarding the RP. Different evidences were found by Dohan Ehrenfest and coworkers (Dohan Ehrenfest, Del Corso, Diss, Mouhyi, & Charrier, 2010) reporting that the type of tested tube (dry glass or glass-coated plastic tubes) did not influence the architecture of PRF.

However, even if PRF is similar to CGF, it is prepared using different devices and technologies. Moreover, the authors (Dohan Ehrenfest

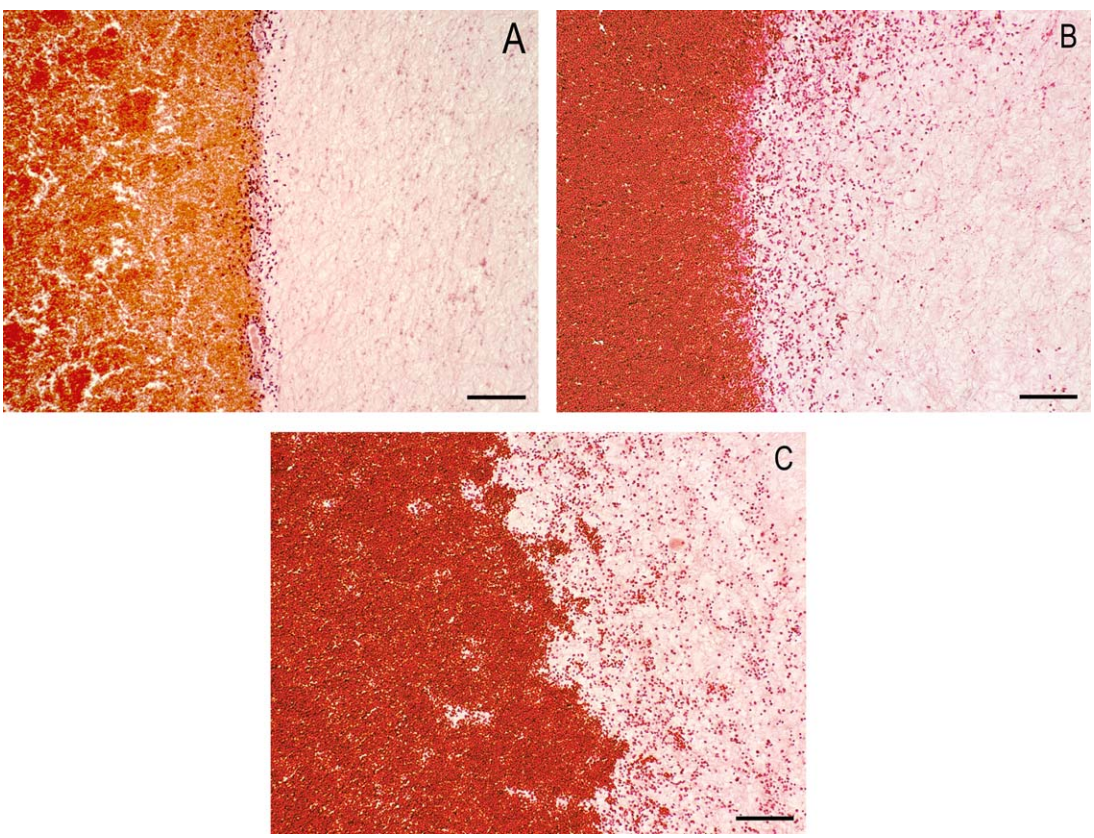

FIGURE 3 Micrographs of CGFs buffy coat stained with HE: (a) plastic silica coated tube, (b) glass round bottom tube, (c) glass conical bottom tube. Bar = 100 μm. [Color figure can be viewed at wileyonlinelibrary.com.]

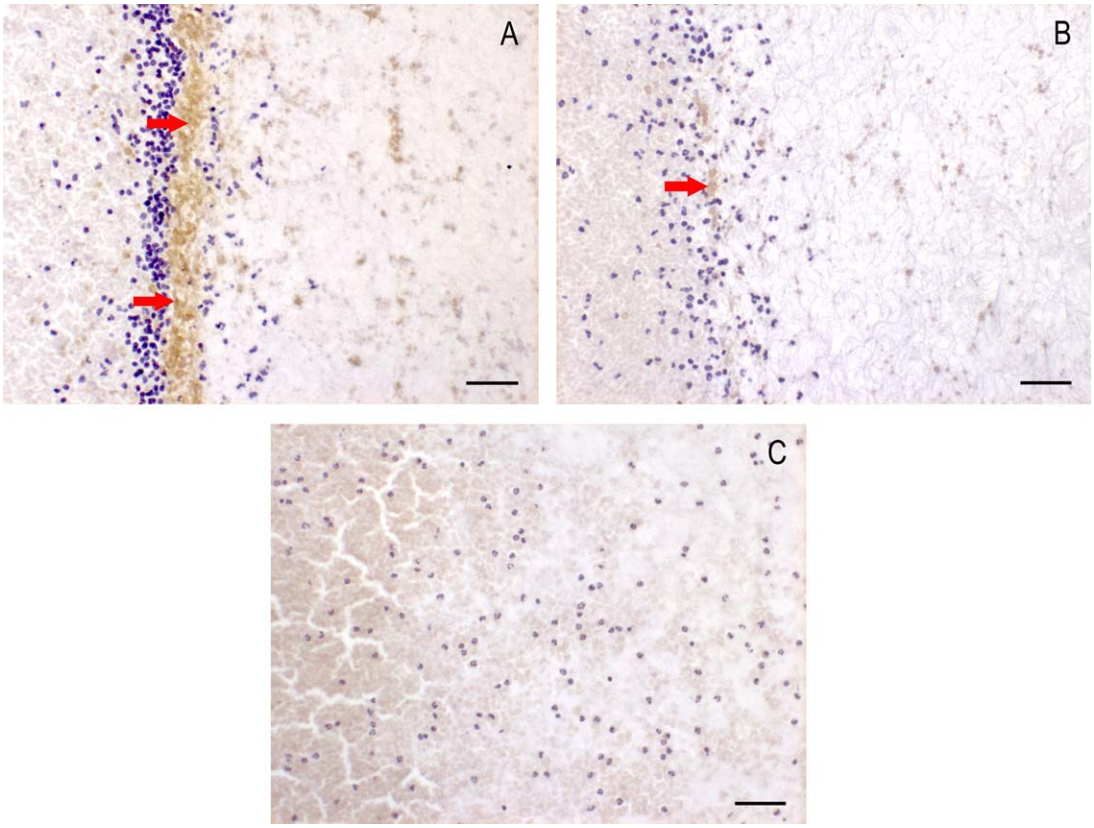

FIGURE 4 CD61 immunostaining for platelets: (a) plastic silica coated tubes, (b) glass round bottom tubes, (c) glass conical bottom tubes. Bar = 40 μm. Red arrows indicate the platelets. [Color figure can be viewed at wileyonlinelibrary.com.]

et al., 2010) did not use a different shape of tubes that we found to be responsible of the different results.

In conclusion, our results show that is sufficient to vary a parameter in the preparation of platelet concentrates (as for example the use of different tubes, as in this case), that the final obtained product is different. These differences could be useful in some specific fields and in particular when we need a large amount of CGF, reducing the volume of blood to be collected, and/or when we need to use strictly the WP, not contaminated by the red blood cells. Some fields of application are, for example, in many dental, oral and maxillofacial surgical procedures (i.e., periapical tissues repair, bone filling of periodontal intrabony defects, alveolar bone regeneration and reconstruction, dental extraction socket preservation, gingival recession treatment, mandibular and maxilla fractures, sinus floor elevation), to promote bone and soft tissues healing and regeneration.

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REFERENCES

Agarwal, K., Agarwal, K., & Kumar, N. (2013). Platelet concentrates: Regenerating the lost tissues. *Journal of Pharmacy and Bioallied Sciences*, *5*, 329–330.

Anitua, E., Pelacho, B., Prado, R., Aguirre, J.J., Sánchez, M., Padilla, S., … Prosper, F. (2015). Infiltration of plasma rich in growth factors enhances in vivo angiogenesis and improves reperfusion and tissue remodeling after severe hind limb ischemia. *Journal of Controlled Release*, *202*, 31–39.

Bettega, G., Brun, J.P., Boutonnat, J., Cracowski, J.L., Quesada, J.L., Hegelhofer, H., … Richard, M.J. (2009). Autologous platelet concentrates for bone graft enhancement in sinus lift procedure. *Transfusion*, *49*, 779–785.

Borsani, E., Bonazza, V., Buffoli, B., Cocchi, M.A., Castrezzati, S., Scarì, G., … Rodella, L. F. (2015) Biological characterization and in vitro effects of human concentrated growth factor preparation: An innovative approach to tissue regeneration. *Biology and Medicine* (Aligarh), *7*, 5.

Chen, Y., Cai, Z., Zheng, D., Lin, P., Cai, Y., Hong, S., … Wu, D. (2016). Inlay osteotome sinus floor elevation with concentrated growth factor application and simultaneous short implant placement in severely atrophic maxilla. *Scientific Report*, *6*, 27348.

Dohan Ehrenfest, D.M., Del Corso, M., Diss, A., Mouhyi, J., & Charrier, J. B. (2010). Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane. *Journal of Periodontology*, *81*, 546–555.

Dohan Ehrenfest, D.M., Rasmusson, L., & Albrektsson, T. (2009). Classification of platelet concentrates: From pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends in Biotechnology*, *27*, 158–167.

Honda, H., Tamai, N., Naka, N., Yoshikawa, H., & Myoui, A. (2013). Bone tissue engineering with bone marrow-derived stromal cells integrated with concentrated growth factor in Rattus norvegicus calvaria defect model. *Journal of Artificial Organs*, *16*, 305–315.

Martínez, C.E., Smith, P.C., & Palma Alvarado, V.A. (2015). The influence of platelet-derived products on angiogenesis and tissue repair: A concise update. *Frontiers in Physiology*, *6*, 290.

Mehta, S., & Watson, J.T. (2008). Platelet rich concentrate: Basic science and current clinical applications. *Journal of Orthopaedic Trauma*, *22*, 432–438.

Naik, B., Karunakar, P., Jayadev, M., & Marshal, V.R. (2013). Role of Platelet rich fibrin in wound healing: A critical review. *Journal of Conservative Dentistry*, *16*, 284–293.

Oryan, A., Alidadi, S., & Moshiri, A. (2016). Platelet-rich plasma for bone healing and regeneration. *Expert Opinion on Biological Therapy*, *16*, 213–232.

Prakash, S., & Thakur, A. (2011). Platelet concentrates: Past, present and future. *Journal of Maxillofacial and Oral Surgery*, *10*, 45–49.

Rodella, L.F., & Bonazza, V. (2015). Platelet preparations in dentistry: How? Why? Where? When? *World Journal of Stomatology*, *4*, 39–55.

Rodella, L.F., Favero, G., Boninsegna, R., Buffoli, B., Labanca, M., Scarì, G., ... Rezzani, R. (2011). Growth factors, CD34 positive cells, and fibrin network analysis in concentrated growth factors fraction. *Microscopy Research and Technique*, *74*, 772–777.

Schär, M.O., Diaz-Romero, J., Kohl, S., Zumstein, M.A., & Nesic, D. (2015). Platelet-rich concentrates differentially release growth factors and induce cell migration in vitro. *Clinical Orthopaedics and Related Research*, *473*, 1635–1643.

Sohn, D.S., Heo, J.U., Kwak, D.H., Kim, D.E., Kim, J.M., Moon, J.W., ... Park, I.S. (2011). Bone regeneration in the maxillary sinus using an autologous fibrin-rich block with concentrated growth factors alone. *Implant Dentistry*, *20*, 389–395.

Yu, B., & Wang, Z. (2014). Effect of concentrated growth factors on beagle periodontal ligament stem cells in vitro. *Molecular Medicine Reports*, *9*, 235–242.