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Research article

Release kinetics of platelet-derived and plasma-derived growth factors from autologous plasma rich in growth factors

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SUMMARY

Many studies have evaluated the biological effects of platelet rich plasma reporting the final outcomes on cell and tissues. However, few studies have dealt with the kinetics of growth factor delivery by plasma rich in growth factors. Venous blood was obtained from three healthy volunteers and processed with PRGF-Endoret technology to prepare autologous plasma rich in growth factors. The gel-like fibrin scaffolds were then incubated in triplicate, in a cell culture medium to monitor the release of PDGF-AB, VEGF, HGF and IGF-I during 8 days of incubation. A leukocyte-platelet rich plasma was prepared employing the same technology and the concentrations of growth factors and interleukin-1β were determined after 24 h of incubation. After each period, the medium was collected, fibrin clot was destroyed and the supernatants were stored at −80 °C until analysis. The growth factor delivery is diffusion controlled with a rapid initial release by 30% of the bioactive content after 1 h of incubation and a steady state release when almost 70% of the growth factor content has been delivered. Autologous fibrin matrix retained almost 30% of the amount of the growth factors after 8 days of incubation. The addition of leukocytes to the formula of platelet rich plasma did not increase the concentration of the growth factors, while it drastically increased the presence of pro-inflammatory IL-1β. Further studies employing an in vitro inflammatory model would be interesting to study the difference in growth factors and pro-inflammatory cytokines between leukocyte-free and leukocyte-rich platelet rich plasma.

1. Introduction

In the 21st century, dentistry has made significant progress in the management of clinical problems thanks to the research in the biomedical fields. Scientists have learned that controlled release of bioactive substances is more efficient in tissue regeneration than simple bolus delivery (Sakiyama-Elbert et al., 2008) as the former ensures their availability over a lengthier period of time and protects them from degradation before optimal outcome is achieved. It is known that tissue healing progressively fabricates and replaces the damaged tissue to restore structure and function ((Werner and Grose, 2003). The first events of tissue healing are inflammatory and, after the first week of injury, tissue building cells reach the injured zone and start synthesizing tissue matrix (Martin, 1997; Werner and Grose, 2003).

Adapting the use of growth factor delivery to dentistry has resulted in the development of protocols benefiting from the autologous growth factors present in platelets and plasma (Anitua, 1999; Marx et al., 1998). Plasma rich in growth factors is an autologous technology using calcium ions to activate growth factor delivery from the platelets and to develop a fibrin network with the aim of promoting tissue regeneration (Anitua et al., 2007). This 100% autologous preparation not only enhances tissue healing but also improves the clinical outcomes of various surgical procedures by minimizing complications like pain, inflammation and morbidity (Rosano et al., 2013).

These desirable outcomes have widened the application of plasma rich in growth factors to clinical practice in dentistry in the enhancement of graft handling, promotion of bone formation, and the generation of autologous fibrin membrane to cover surgical sites (Anitua et al., 2012a). Clinical applications range from simple post-extraction socket treatment to sinus floor elevation, vertical and horizontal bone augmentation as well as the enhancement of dental implant osseointegration (Anitua et al., 2012b, 2013). Furthermore, this autogenous biological therapy has been used to promote periodontal tissue regeneration in infrabony defects, an adjuvant in the treatment of gingival recessions (Del Fabbro et al., 2011), and showed efficiency in the treatment and prevention of bisphosphonate-related osteonecrosis of the jaws (Curi et al., 2007; Mozzati et al., 2012).

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The biological mechanism that mediates the effect of platelet rich plasma involves the release of growth factor to the surrounding milieu where it binds to specific receptors on the cell membrane (James and Bradshaw, 1984). However, the majority of studies available on the use of platelet rich in growth factors only describe the final outcome at cellular and tissue levels without addressing the kinetics of growth factor release from the fibrin scaffold.

Platelet rich plasma could be categorized into two main types: leukocyte-free platelet rich plasma and leukocyte-platelet rich plasma (Leukocyte-PRP) (McCarrel et al., 2012). The presence of leukocytes has been proposed as an additional source of growth factors and of antimicrobial properties (Castillo et al., 2011; Filardo et al., 2012). At the same time, leukocytes, on the other hand, release pro-inflammatory cytokines that could increase the inflammation process and counterbalance the positive effect on tissue regeneration (Castillo et al., 2011; McCarrel et al., 2012). To elucidate the potential effect of leukocytes on growth factor and cytokine release from platelet rich plasma, we selected PRGF-Endoret technology primarily due to its flexibility in incorporation or exclusion of leukocytes in the final platelet rich plasma preparation. This enables us to use the same platelet enriching factor to produce two preparations, solely differing in the presence or absence of leukocytes.

Therefore, the aim of the present study has been to characterize growth factor release from fibrin scaffold obtained with PRGF-Endoret technology and to determine the effect of adding the buffy coat to the fibrin scaffold in relation to the dose of the released growth factors and pro-inflammatory cytokines. Two platelet derived growth factors, platelet-derived growth factor (PDGF-AB) and vascular endothelial growth factor (VEGF), and two plasmatic growth factors, hepatocyte growth factor (HGF) and insulin-like growth factor (IGF-I), together with a relevant pro-inflammatory cytokine (interleukin -1β; IL-1β) were analyzed.

2. Materials and methods

Plasma rich in growth factors (PRGF) was prepared according to manufacturer's instructions (Biotechnology Institute, Vitoria, Spain). For this purpose, 9 ml of venous blood was collected in a tube containing 0.9 ml of 3.8% of sodium citrate as anticoagulant from 3 volunteers who gave a total of 19 tubes each. The citrated whole blood was centrifuged at 580 *g* for 8 min (PRGF-Endoret System, Biotechnology Institute, Spain). For each tube, the 2 ml of PRGF just above the buffy coat was collected and used for the study of growth factor release from PRGF clot. Leukocyte-PRP was prepared by additionally incorporating the buffy coat into the platelet rich fraction. To specify the composition of platelet rich plasma, whole blood and the obtained plasma fractions were analyzed using a Micros 60 hematology analyzer (Horiba ABX S.A.S, Montpelier, Herault, France).

The morphological analysis of PRGF clot was realized with scanning electron microscopy (SEM) after sample fixation in 2.5%-glutaraldehyde, dehydration in ascending ethanol concentrations and gold-sputtering after critical-point drying.

The kinetics of PDGF-AB, VEGF, HGF, IGF-I and Interleukin-1β (IL-1β) release from PRP clots were characterized in triplicate by incubation of the PRP clot with Osteoblast cell medium (ObM) without growth supplements (ScienCell Research Laboratories, Carlsbad, California, USA) for 5 and 24 h and 3, 6, 7 and 8 days. In each well of a 12-well cell culture plate, 1.1 ml of PRP was activated with 55 μl of 10%CaCl2 solution (PRGF activator) and incubated for 20–30 min at 37 °C for clot formation. The clot thickness was about 5 mm. Thereafter, 1.6 ml of the osteoblast cell medium was added to each well and then the cell culture plates were 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. The PRP clot was then placed in 5 ml tubes and mixed with 800 μl of ObM medium. The mixture was then homogenized at 20,000 rpm for 2 min using a Polytron extraction-dispersing machine (Kinematica AG, Lucerne, Lucerne, Switzerland). After homogenization, the suspension was centrifuged at 21,000 *g* for 5 min at 4 °C and the supernatant was stored at −80 °C until analysis.

The release of growth factors form Leukocyte-PRP was evaluated after 24 h of incubation following the previously-described methodology. The increase in the growth factors content was expressed by calculating the percentage of growth factor concentration in Leukocyte-PRP to its concentration in PRGF after 24 h of incubation.

Quantification of growth factors was performed using ELISA kits for PDGF-AB, VEGF, HGF, IGF-I and IL-1β according to the manufacturer's protocol (R&D Systems Inc, Minneapolis, Minnesota, USA). This quantification was performed for the two supernatants recovered after each time period.

The kinetics of in vitro release of growth factors was fitted up to the first 60% using Korsemeyer-Peppas equation (power law)

$$M(\%) = k.t^n \quad (1)$$

where M is the cumulative bioactive release at time t, the release exponent is n, and k is a constant incorporating structural and geometric characteristics of the matrix. For cylindrical geometry, the value $n = 0.45$ indicates diffusion-controlled release (Fickian diffusion), $n = 0.89$ swelling-controlled release (Case-II transport), and n values between 0.45 and 0.89 indicates superposition of both phenomena (anomalous transport).

3. Results

3.1. Composition of platelet rich preparations

The PRGF-Endoret preparation protocol resulted in a platelet enrichment factor of 2.5 ± 0.50 for PRGF and 2.8 ± 0.32 for Leukocyte-PRP. The leukocyte content in the PRGF was $0.3 \times 10^3/\mu l$ while the Leukocyte-PRP contained $5.7 \times 10^3/\mu l$. These results demonstrate that both preparations differ only in leukocyte concentration as similar platelet concentrations were obtained. The morphological analysis of the fibrin scaffold (without leukocytes) obtained showed the fibrillar nature of the fibrin mesh and the formation of a highly porous scaffold as a result of cross-linking of fibers (Fig. 1).

3.2. Growth factors release from PRGF scaffold

The first remark of the study of growth factors release is the physiological variation in the concentration of growth factors (Table 1). The highest content was measured for IGF-I followed by the PDGF-AB, VEGF and HGF.

Initially, the release of Platelet derived growth factor (PDGF) was measured. The initial burst effect was slowed to a plateau of almost 70% of the total dose after 3 days of incubation. Interestingly, the PRGF clot retained about 30% of the PDGF-AB released by the platelets after 8 days of incubation (Fig. 2). The release pattern of this growth factor was similar for both patients. The fitting of the release data to power law have indicated diffusion as the mechanism by which PDGF-AB is released to the incubation medium (Table 1).

Differently from PDGF-AB, the content of VEGF in the PRGF clot varied in the samples obtained from the three patients (Table 1). This variation was reflected in the release pattern of VEGF for all volunteers. The initial release of VEGF for the three patients was equally rapid, reaching the release rate of about 70% of the total

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