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Article in Clinical Oral Investigations · February 2017
DOI: 10.1007/s00784-017-2063-9

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Injectable platelet rich fibrin (i-PRF): opportunities in regenerative dentistry?

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Received: 31 August 2016 / Accepted: 25 January 2017
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Abstract

Objectives Platelet rich plasma (PRP) has been utilized in regenerative dentistry as a supra-physiological concentrate of autologous growth factors capable of stimulating tissue regeneration. Despite this, concerns have been expressed regarding the use of anti-coagulants, agents known to inhibit wound healing. In this study, a liquid formulation of platelet rich fibrin (PRF) termed injectable-PRF (i-PRF) without the use of anti-coagulants was investigated.

Materials and methods Standard PRP and i-PRF (centrifuged at 700 rpm (60G) for 3 min) were compared for growth factor release up to 10 days (8 donor samples). Furthermore, fibroblast biocompatibility at 24 h (live/dead assay); migration at 24 h; proliferation at 1, 3, and 5 days, and expression of PDGF, TGF-β, and collagen1 at 3 and 7 days were investigated.

Results Growth factor release demonstrated that in general PRP had higher early release of growth factors whereas i-PRF showed significantly higher levels of total long-term release of PDGF-AA, PDGF-AB, EGF, and IGF-1 after 10 days. PRP showed higher levels of TGF-β1 and VEGF at 10 days. While both formulations exhibited high biocompatibility and higher fibroblast migration and proliferation when compared to control tissue-culture plastic, i-PRF induced significantly highest migration whereas PRP demonstrated significantly highest cellular proliferation. Furthermore, i-PRF showed significantly highest mRNA levels of TGF-β at 7 days, PDGF at 3 days, and collagen1 expression at both 3 and 7 days when compared to PRP.

Conclusions i-PRF demonstrated the ability to release higher concentrations of various growth factors and induced higher fibroblast migration and expression of PDGF, TGF-β, and collagen1. Future animal research is now necessary to further validate the use of i-PRF as a bioactive agent capable of stimulating tissue regeneration.

Clinical relevance The findings from the present study demonstrate that a potent formulation of liquid platelet concentrates could be obtained without use of anti-coagulants.

Keywords Fibrin · Blood platelets · Regeneration · Wound healing · Fibroblasts · Platelet rich fibrin

Introduction

Platelet concentrations have been utilized in dentistry for over three decades as a regenerative tool capable of releasing supra-physiological doses of growth factors responsible for inducing tissue regeneration derived from autologous sources [1, 2].
Since then, platelet rich plasma (PRP) was developed having widespread use not only in regenerative dentistry but also in maxillofacial surgery, orthopedic surgery, and esthetic medicine [3–7]. Despite this, various concerns have been raised including the use of bovine thrombin and various other anti-coagulants, known suppressors of tissue regeneration [3, 8, 9]. For these reasons, platelet rich fibrin (PRF) was developed as a first source of autogenous blood-derived growth factors harvested without the use of anti-coagulants [10]. PRF therefore forms a three-dimensional fibrin matrix that may further serve as a scaffold for tissue regeneration by bearing the feature of acting as a barrier membrane in guided bone and tissue regeneration (GBR, GTR) procedures while simultaneously holding a number of growth factors responsible for wound healing [11–13].

Over the past decade [10], PRF has gained tremendous momentum having been utilized for a variety of dental and medical procedures. In the dental field, PRF has been utilized for the treatment of extraction sockets [14–17], gingival recessions [18–20], palatal wound closure [21–23], regeneration of periodontal defects [24], and hyperplastic gingival tissues [25]. In other medical fields, PRF has been utilized for the successful management of hard-to-heal leg ulcers including diabetic foot ulcers, venous leg ulcers, and chronic leg ulcers [26]. Furthermore, hand ulcers, facial soft tissue defects, laparoscopic cholecystectomy, deep nasolabial folds, facial defects, superficial rhytids, acne scars, lipostructure surgical procedures, chronic rotator cuff tears, and acute traumatic ear drum perforations have also all been treated with PRF [26]. Reported advantages include faster wound healing, faster angiogenesis, low costs, and complete immune-biocompatibility [27–30]. Therefore, while initial and early experiments revealed that up to six to eight times higher than normal blood concentrations could be reached with PRP, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor-beta1 (TGF-β1) [31], its use includes anti-coagulants which have also been reported to inhibit wound healing and research has since aimed to develop new protocols without the use of anti-coagulants. Very recently, Anitua et al. proposed removing anti-coagulant agents altogether from platelet rich in growth factor (PRGF) formulations following approximately 20 years of research utilizing such therapies [32].

In this study, the development of an injectable formulation of PRF (termed i-PRF) has been pursued with the aim of delivering to clinicians an easy to use platelet concentrate in liquid formulation which can be either utilized alone or combined easily with various biomaterials. Taking advantage of slower and shorter centrifugation speeds, a higher presence of regenerative cells with higher concentrations of growth factors can be observed when compared to other formulations of PRF utilizing higher centrifugation speeds as highlighted by our group’s previous research [33, 34]. The aim of the present study was to compare i-PRF to the clinically utilized PRP for total growth factor release as well as on human gingival fibroblast cell biocompatibility and activity. Cells were cultured with PRP or i-PRF and investigated for cell migration, proliferation, and messenger RNA (mRNA) levels of growth factors (PDGF and TGF-β1) as well as collagen1 mRNA levels in vitro.

Materials and methods

Preparation of PRP and i-PRF

Blood samples were collected with the informed consent of 6 volunteer donors (12 total samples), and blood was then processed for PRP and i-PRF preparation. All blood samples were obtained from members of our laboratory between the ages of 30 and 60. PRP platelet concentration was prepared via a protocol as previously described [35]. Briefly, 10 ml of whole blood with adding 1.0 ml of acid citrate dextrose (ACD) solution (Sigma, St. Louis, MO, USA) was centrifuged at 1000 rpm (123×g) for 7 min at room temperature. The upper layer withuffy coat was transferred to a new, sterile centrifuge tube. The second spin was performed at 3000 rpm (1107×g) for 10 min at room temperature, and finally, the PRP was collected (Duo Centrifuge, Process for PRF, Nice, France). For i-PRF preparation, two tubes of 10 ml of whole blood without anticoagulant were centrifuged at 700 rpm for 3 min (60×g) at room temperature by a Duo Centrifuge (Process for PRF, Nice, France). The upper liquid layer was collected as i-PRF. Collected PRP and i-PRF samples were transferred to six-well plastic culture dishes with 5 ml of culture media (DMEM; Gibco, Life technologies, Carlsbad, CA, USA) and processed as further described.

Protein quantification with ELISA

In order to determine the amount of released growth factors (GFs) from PRP and i-PRF at 15 min, 60 min, 8 h, 1 day, 3 days, and 10 days, samples were placed into a shaking incubator (60 rotations per minute) at 37 °C to allow for GF release into the culture media. At each time point, 5 ml of culture media was collected, frozen at −20 °C, and replaced with 5 ml of additional culture media. Protein quantification was carried out using ELISA. At designated time points, PDGF-AA (DY221, range = 15.60–1000 pg/ml), PDGF-AB (DY222, range = 15.60–1000 pg/ml), PDGF-BB (DY220, 31.20–2000 pg/ml), TGF-β1 (DY240, range = 31.20–2000 pg/ml), VEGF (DY293B, range = 31.20–2000 pg/ml), EGF (DY236, range = 3.91–250 pg/ml), and IGF-1 (DY291, range = 31.20–2000 pg/ml) were quantified using an ELISA kit (DuoSet, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol as previously described.
Absorbance was measured at 450 and 570 nm using a DTX880 microplate reader (Beckman Coulter, Brea, CA, USA) and subtracted at 570 nm from the readings at 450 nm. All samples were measured in duplicate, and three independent experiments were performed for each platelet concentrate.

**Cell culture**

Platelet concentrates including PRP and i-PRF were incubated for 3 days in a spinning chamber at 37 °C, and thereafter, conditioned media were collected and utilized in future experiments as 20% of the total volume. Human gingival fibroblasts (HGF-1) were purchased from ATCC (Manassas, VA, USA). All cells were detached from tissue culture plastic using 0.25% EDTA-trypsin (Gibco) prior to reaching confluency. Cells used for experimental seeding were from passages 4–6 as previously described. Cells were cultured in a humidified atmosphere at 37 °C in a growth medium consisting of DMEM (Gibco), 10% fetal bovine serum (FBS; Gibco), and 1% antibiotics (Gibco). Media was changed two times per week. Cells were seeded with 20% conditioned media from PRP and i-PRF contained within a growth medium at a density of 10,000 cells for cell viability and proliferation experiments and 50,000 cells for real-time PCR per well in 24-well plates. Standard tissue culture plastic (TCP) was utilized as a control.

**Cell viability assay**

At 24 h post cell seeding, cells were evaluated using a live-dead staining assay according to the manufacturer’s protocol (Enzo Life Sciences AG; Lausen, Switzerland). Fluorescent images were quantified with an inverted fluorescent microscope (IX51, OLYMPUS, Tokyo, Japan). Thereafter, cells were expressed as percentages of live versus dead cells following cell culture growth with TCP, PRP, and i-PRF. The experiments were performed in triplicate with three independent experiments performed.

**Cell migration assay**

The migration assay was performed using 24-well plates and polyethylene terephthalate cell culture inserts with a pore size of 8 μm (Falcon, Coming Inc., Corning, NY, USA). The 20% platelet conditioned media in DMEM containing 10% FBS were filled into the lower compartment of the wells. After being starved in DMEM containing 0.5% FBS for 12 h, 10,000 resuspended cells were seeded in the upper compartment. After 24 h, cells were fixed with 4% formaldehyde for 2 min. Thereafter, cells were permeabilized by acetone (Sigma) for 15 min and stained with hematoxylin solution (Sigma) for 20 min. The upper side of the filter membrane were rinsed and gently wiped by a cotton swab to remove the cell debris. The numbers of cells on the lower side of the filter were counted under a microscope. The experiments were performed in triplicate with three independent experiments performed.

**Proliferation assay**

Cells were quantified using an MTS colorimetric assay (Promega, Madison, WI, USA) at 1, 3, and 5 days for cell proliferation as previously described [36]. At desired time points, cells were washed with phosphate-buffered saline (PBS) and quantified using a DTX880 microplate reader. The experiments were performed in triplicate with three independent experiments performed.

**Real-time PCR analysis**

Total RNA was harvested at 3 and 7 days post stimulating for HGF-1 cells to investigate mRNA levels of TGF-β, PDGF, and collagen1a2 (COL1a2). Primer and probe sequences for genes were fabricated with primer sequences according to Table 1. RNA isolation was performed using High Pure RNA Isolation Kit (Roche, Basel, Switzerland). Real-time RT-PCR was performed using Roche Master Mix and quantified on the StepOne™ plus Real time PCR system from Applied Biosystems (Foster City, CA, USA). The ΔΔCt method was used to calculate gene expression levels normalized to the expression of GAPDH. The experiments were performed in triplicate with three independent experiments performed.

**Statistical analysis**

All experiments were performed in triplicate with three independent experiments for each condition. Means and standard errors (SE) were calculated, and data were analyzed for statistical significance using one-way analysis for cell viability and migration assay, two-way analysis of variance for ELISA, proliferation assay, and real-time PCR analysis with Tukey test (*p values <0.05 were considered significant) by GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**Growth factor release from PRP and i-PRF**

In a first set of experiments, the release of growth factors from PRP and i-PRF was investigated by ELISA including PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, EGF, and IGF-1 (Figs. 1 and 2). Interestingly, all growth factors investigated demonstrated a significantly higher early (15 min) release of
growth factor from PRP when compared to i-PRF (with the exception of IGF, Figs. 1 and 2). Thereafter, the total release of growth factors was quantified up to a 10-day period (Figs. 1 and 2). It was found that PDGF-AA, PDGF-AB, EGF, and IGF-1 all demonstrated higher total growth factors released from i-PRF when compared to PRP. Interestingly, however, total growth factor release of PDGF-BB, VEGF, and TGF-β1 were significantly higher in PRP when compared to i-PRF (Figs. 1f and 2b, d). These results point to the fact that various spin protocols/cell types found in PRP/i-PRF are likely responsible for the variations as discussed later.

**Biocompatibility of PRP and i-PRF on human gingival fibroblasts**

In the first cell culture experiment, the effects of PRP and i-PRF were investigated on cell viability of human gingival fibroblasts. It was found that both PRP and i-PRF demonstrated excellent cell cytocompatibility by demonstrating most notably high living cells (green cells, Fig. 3) with very few observable dead cells (red cells). It was therefore concluded that both PRP and i-PRF were fully biocompatible under the present in vitro cell culture model (Fig. 3).

**Table 1** List of primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>hTGF-β F</td>
<td>actactacgccaaggaggtcac</td>
</tr>
<tr>
<td>hTGF-β R</td>
<td>tggtaacagtctagatgttgc</td>
</tr>
<tr>
<td>hPDGF F</td>
<td>cacaccctctcttgtgtatat</td>
</tr>
<tr>
<td>hPDGF R</td>
<td>gttatcgttaaatgctcatcc</td>
</tr>
<tr>
<td>hCOL1a2 F</td>
<td>cgggccagcaagctggtagtgg</td>
</tr>
<tr>
<td>hCOL1a2 R</td>
<td>ggcgcagcattgatggttcc</td>
</tr>
<tr>
<td>hGAPDH F</td>
<td>agcagactgcacac</td>
</tr>
<tr>
<td>hGAPDH R</td>
<td>gccaataegccaaatce</td>
</tr>
</tbody>
</table>

Fig. 1 ELISA protein quantification at each time point of (a) PDGF-AA, e PDGF-AB, and e PDGF-BB over a 10-day period. Total accumulated growth factor released over a 10-day period for b PDGF-AA, d PDGF-AB, and f PDGF-BB (*p < 0.05 signifies significant difference between groups, and *p < 0.05 signifies significantly higher than the other group).
Influence of PRP and i-PRF on human gingival fibroblast activity

Following confirmation of high cell survival in the biocompatibility assays, PRP and i-PRF were then investigated on gingival fibroblasts migration, proliferation, and mRNA expression of TGF-β, PDGF, and COL1a2 (Figs. 4 and 5). It was first found that while PRP induced a 180% increase in cell migration when compared to control TCP, a significantly higher increase was observed when cells were cultured with i-PRF (Fig. 4a). It was found that a 270% increase was observed when fibroblasts were cultured with i-PRF, significantly higher than all other groups (Fig. 4a). Thereafter, it was found that PRP induced significantly higher cell numbers at
3 days when compared to control TCP (Fig. 4b). Furthermore, while both PRP and i-PRF were able to significantly increase cell numbers at 5 days when compared to control TCP, PRP demonstrated significantly higher values when compared to all other groups (Fig. 4b).

Investigation of mRNA levels revealed that only i-PRF was able to significantly upregulate TGF-β at 7 days post seeding when compared to TCP and PRP (Fig. 5a). Similarly, only i-PRF was able to significantly increase mRNA levels of PDGF at 3 days (Fig. 5b). Analysis of COL1a2 mRNA levels revealed that while PRP was able to induce a significant increase at 7 days when compared to TCP, i-PRF demonstrated the significantly highest levels at both 3 and 7 days when compared to both PRP and TCP (Fig. 5c).

Discussion

The use of regenerative modalities in dentistry has become a standard of care for many clinicians working in the field of implant dentistry. Currently, a variety of biomaterials are routinely being utilized including barrier membranes, bone grafting materials, and bioactive growth factors to facilitate new tissue regeneration. Noteworthy, dimensional changes of alveolar bone following tooth loss have remained a prominent challenge for the clinician and a variety of regenerative procedures have since been utilized [37–40]. One such proposed method has been the use of platelet concentrates including PRP and PRF which both utilize supra-physiological doses of autologous growth factors derived from the patient’s own blood further capable of speeding tissue regeneration [34, 35]. Despite their widespread use, concerns have been expressed regarding the use of anti-coagulants in PRP [32] which was initially added to centrifugation protocols in order to maintain the liquid consistency of PRP to facilitate biomaterial mixing. On the other hand, initial PRF formulations lacked a liquid concentrate of proteins as standardized PRF contains the majority of its growth factor concentration encapsulated within its fibrin matrix. For these reasons, major development and advancements were recently made with the aim of developing a liquid formulation of PRF which does not contain any anti-coagulants or fibrin matrix.

These advancements were made possible due to the recent findings by Ghanaati et al. that introduced the “low-speed concept” for blood centrifugation whereby lower centrifugation speeds were shown to contain higher numbers of cells including leukocytes prior to the formation of a fibrin clot [33]. Leukocytes are immune cells having vast importance in tissue regeneration by directing and recruiting various cell types during the wound healing process [41–43]. Since high centrifugation forces during the fabrication of PRP or PRF are known to shift cell populations from the top of centrifugation tubes towards the bottom, it was recently hypothesized that by reducing centrifugation G-force, a total increase in leukocyte numbers would remain in the top third layer of platelet concentrate tubes where PRP and PRF are collected [33].
Furthermore and in agreement with this hypothesis, the added number of cells contained within this fibrin matrix (now termed advanced PRF) was further shown to release higher total growth factor release of PDGF, TGF-β, VEGF, EGF, and IGF when compared to control L-PRF [34, 35]. Interestingly, the release of growth factors from PRP and i-PRF followed different trends also (Figs. 1 and 2). Therefore, it may be hypothesized that the differences in spin protocols are suggested to have collected slightly different cell populations and/or total growth factors responsible for the variations in growth factor release over time (Figs. 1 and 2). Another interesting finding was the fact that while PRP was slowly dissolved over time, i-PRF formed a small clot likely as a result of fibrin components that acted as a dynamic gel with cells likely contained within its hydrogel. It is therefore hypothesized that even following 10 days, an additional release of growth factors could still be expected from i-PRF whereas PRP had basically dissolved entirely after 10 days. Future research to further clarify the exact reasons for these differences remains necessary.

In the future, there also remains great interest to continuously and steadily increase our understanding of platelet concentrates and the role of the various cell types found within their formulations. It remains an important challenge for researchers working in regenerative dentistry to further characterize the potential of each platelet formulation on new bone formation and tissue wound healing and to further compare their regenerative potential by fully revealing their added advantages/disadvantages. Nearly 20 years ago, Anutia et al. developed the platelet rich in growth factor (PRGF) for regenerative dentistry [44] and has since been researching new protocols to avoid the use of anti-coagulants [32]. Currently, there is a need to further speed the development of liquid platelet concentrates while simultaneously avoiding the use of unnecessary anti-coagulants or other non-autogenous additions to their formulations which may prevent wound healing. Therefore, animal and clinical studies investigating the ability for i-PRF to improve wound healing and new bone formation for future clinical benefit remain necessary.

**Conclusion**

In summary, the results from the present study demonstrate that both PRP and i-PRF showed the potential to contain a number of growth factors responsible for tissue regeneration capable of inducing fibroblast behavior. Interestingly, it was found that variability in growth factor release as well as cellular effects were found between PRP and i-PRF likely as a result of the different centrifugation protocols/addition of anti-coagulants. Future animal and human research investigating specifically the use of liquid formulation of blood concentrates is necessary to further characterize their advantages in regenerative dentistry for potential clinical benefit.
References


