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Effects of an injectable platelet-rich fibrin on osteoblast behavior and bone tissue formation in comparison to platelet-rich plasma

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Abstract
Platelet-rich plasma (PRP) has been utilized for many years as a regenerative agent capable of inducing vascularization of various tissues using blood-derived growth factors. Despite this, drawbacks mostly related to the additional use of anti-coagulants found in PRP have been shown to inhibit the wound healing process. For these reasons, a novel platelet concentrate has recently been developed with no additives by utilizing lower centrifugation speeds. The purpose of this study was therefore to investigate osteoblast behavior of this novel therapy (injectable-platelet-rich fibrin; i-PRF, 100% natural with no additives) when compared to traditional PRP. Human primary osteoblasts were cultured with either i-PRF or PRP and compared to control tissue culture plastic. A live/dead assay, migration assay as well as a cell adhesion/proliferation assay were investigated. Furthermore, osteoblast differentiation was assessed by alkaline phosphatase (ALP), alizarin red and osteocalcin staining, as well as real-time PCR for genes encoding Runx2, ALP, collagen1 and osteocalcin. The results showed that all cells had high survival rates throughout the entire study period irrespective of culture-conditions. While PRP induced a significant 2-fold increase in osteoblast migration, i-PRF demonstrated a 3-fold increase in migration when compared to control tissue-culture plastic and PRP. While no differences were observed for cell attachment, i-PRF induced a significantly higher proliferation rate at three and five days when compared to PRP. Furthermore, i-PRF induced significantly greater ALP staining at 7 days and alizarin red staining at 14 days. A significant increase in mRNA levels of ALP, Runx2 and osteocalcin, as well as immuno-fluorescent staining of osteocalcin was also observed in the i-PRF group when compared to PRP. In conclusion, the results from the present study favored the use of the naturally-formulated i-PRF when compared to traditional PRP with anti-coagulants. Further investigation into the direct role of fibrin and leukocytes contained within i-PRF are therefore warranted to better elucidate their positive role in i-PRF on tissue wound healing.

Introduction
Platelet-rich plasma (PRP) was first utilized as a regenerative agent derived from autologous blood (platelet concentrates). Its use in modern medicine has exponentially increased throughout many fields including oral and maxillofacial surgery where, Marx et al. utilized this therapy either alone or combined with bone marrow aspirates [1]. Since PRP is known to contain a 6–8-fold increase in blood-derived growth factors that influence cellular growth, morphogenesis and differentiation, it has been reported that PRP facilitates bone and tissue healing [2–8]. In the dental field alone, many studies have pointed to the positive effects of PRP during guided bone regeneration [9], early bone formation around implants [10], in combination with bone grafts for maxillary sinus floor augmentation procedures [11] and for the regeneration of periodontal intrabony or furcation defects [12]. Despite its growing popularity and use in the early 1990s, concerns over the additional use of anti-coagulants such as bovine-derived thrombin (amongst others) has been shown to negatively impact wound healing by preventing clot formation [13]; an essential step for the healing of all tissues.

Platelet-rich fibrin (PRF) was therefore developed as a second generation autologous platelet concentrate without the use of anti-coagulants or other additives, which was first termed by Choukroun et al. in 2001 [14]. Recently the effects of PRF have been documented in several systematic reviews demonstrating its long-term effects on tissue-wound healing [15–19]. Two of the main documented advantages of PRF include the fact that it contains host immune defense cells (leukocytes) which act to fight infection [20]. Furthermore, PRF was initially developed with high centrifugation speeds allowing a fibrin clot to form which may be utilized as a three-dimensional scaffold to further speed the healing of bone and gingival tissues [14, 21]. Interestingly, over a decade has since past since PRF was developed and many clinicians now point to the potential use of a liquid version of PRF. Like PRP, a liquid-gel-like consistency allows the biomaterial to be utilized when combined with various biomaterials or
as a sole injection material much like PRP for chondrogenesis in osteoarthritic knees or rotator cuff repair [15, 22, 23]. In 2014, a liquid injectable-platelet-rich fibrin (i-PRF) was developed by modifying spin centrifugation forces. At lower centrifugation speeds and by utilizing non-glass centrifugation tubes, the fibrin coagulation could be slowed down at early time points thus generating an injectable-PRF. Much like traditional PRF, i-PRF contains an increase in leukocyte number and is further able to stimulate growth factor release (unpublished data by our group currently under review).

The aim of this cell study was to compare both PRP and i-PRF on osteoblast behavior. To the best of our knowledge, this is the first study investigating primary human osteoblasts on cell viability, migration, adhesion, proliferation, differentiation and mineralization when cultured with i-PRF.

Materials and methods
Preparation of PRP and iPRF
Blood samples were collected from researchers within our laboratory. PRP was prepared according to previously described protocols [24]. Briefly, 10 ml of whole blood with anti-coagulant (EDTA) was centrifuged at 900g for 5 minutes to remove red blood cell (RBC). Then, a second centrifugation at 2000g for 15 minutes was processed to separate PRP from PPP. Finally, 1ml PRP was obtained. For i-PRF preparation, 10 ml of whole blood without anti-coagulant was centrifuged at 700 rpm for 3 minutes by a duo-Centrifuge (Process for PRF, Nice, France). 1 ml of i-PRF was collected from the upper layer. The collected PRP and i-PRF were then transferred to six well in vitro plastic culture dishes with 5 ml of alpha-minimum essential medium (α-MEM; HyClone, Thermo Fisher Scientific Inc) and processed as further described.

Isolation of human osteoblasts
Human primary osteoblasts were derived from cancellous bone collected from young patient who had undergone implant surgery as previously described in Ref. [25]. Ethical approval and consent forms were signed by all patients for the isolation of osteoblasts. Collected cancellous bone was washed three times with phosphate buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.2) containing 1% antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin, HyClone, Thermo Fisher Scientific Inc). The bone tissue fragments were then transferred into T25 tissue culture flasks with α-MEM containing 20% fetal bovine serum (FBS; Gibco, Life Technologies Corporation) and 1% antibiotics in an incubator at 37°C in a humidified atmosphere containing 5% CO₂. When cells reached confluency, cells were trypsinized and passaged in α-MEM containing 10% FBS. Human osteoblasts used for experimental analysis were utilized from passages 2–5.

Cell culture
Platelet concentrates including PRP and i-PRF were incubated for three days in a humidified 5% CO₂ atmosphere at 37°C and thereafter conditioned media was collected and utilized in future experiments as 20% of the total volume as previously described [26]. Human osteoblasts were cultured in a humidified atmosphere at 37°C in growth medium consisting of α-MEM, 10% FBS and 1% antibiotics. For osteoblasts differentiation, osteogenic differentiation medium (ODM) consisting of α-MEM, 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) were used as previously described in Ref. [27]. Cells were seeded with 20% conditioned media from PRP and i-PRF at a density of 10,000 cells for cell viability, cell migration, cell adhesion and proliferation experiments in growth medium, and 50,000 cells for real-time PCR, ALP, alizarin red and osteocalcin immunofluorescent staining in osteogenic differentiation medium. For experiments lasting longer than five days, medium was replaced twice a week.

Cell viability
At 24 h post cell seeding, human osteoblasts were evaluated using a live-dead staining assay. Live cells were stained with 2µmol/L Calcein-AM (Dojindo, Japan) and dead cells were stained with 4µmol/L propidium iodide (Sigma). The cells were washed with PBS and live/dead reagents were added and incubated for 15 min at 37°C. Fluorescent images were quantified with an Olympus DP71 fluorescence microscope (Olympus Co., Japan). Thereafter, cells were expressed as percentages of live versus dead cells following cell culture growth with PRP and i-PRF.

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 (Costar, Corning Inc., Corning, NY, USA). The 20% platelet conditioned medium in α-MEM with 10% FBS were added into the lower compartment of the wells. After starving the cells in α-MEM containing 0.5% FBS for 12 h the night before, 10,000 cells were resuspended and seeded in the upper compartment and allowed to migrate. After 24 h, cells were fixed with 4% formaldehyde for 15 min and stained with 0.1% crystal violet solution (GoodBio Technology Co., Ltd, Wuhan, China) for 10 min. The upper side of the filter membrane were rinsed and gently wiped by a cotton swab to remove the cell debris. Images on the lower side of the filter were captured under an Olympus DP71 fluorescence microscope (Olympus Co., Japan).

Cell adhesion and proliferation assays
For cell adhesion assay, human osteoblasts were cultured in 24-well plates at a density of 10,000 cells per well for 2, 4 and 8 h. Cell number was counted by staining cells with 4’,6-diamidino-2-phenylindole (DAPI) as previously described [28]. At 2, 4 and 8 h, cells were washed using PBS solution to remove non-attached cells and fixed in 4% formaldehyde for 10 min followed by staining with DAPI. Images were captured on an Olympus DP71 fluorescence microscope. Ten fields of view were captured per sample and nuclei were counted using Image J software as previously described [29].

For the cell proliferation assay, human osteoblasts were seeded in 24-well plates at a density of 10’000 cells per well with 20% culture medium from PRP, i-PRF. At time points 1,3 and five days, osteoblast number was determined by the Cell Counting Kit-8 (Dojindo, Japan) and measured by a microplate reader scanning at 450 nm (PowerWave XS2, BioTek, Winooski, VT, USA). Samples were performed in triplicate with 3 independent experiments performed.

ALP activity assay
Human osteoblasts were seeded in 24-well plates at a density of 50,000 cells per well in osteogenic differentiation medium. At a time point of seven days, cells were solubilized in 0.1% Triton X-100 at 4°C. After sonication and centrifugation, ALP activity in the supernatant was measured as 3[OD405nm/OD562nm]. Samples were performed in triplicate with three independent experiments. For ALP staining, human osteoblasts were fixed 4% formaldehyde for 15 min. Alkaline dye mixture were prepared by mixing 5ml 0.2M Tris–HCl buffer (pH = 8.5), 0.05mol N,N-Dimethyl Formamid (Amresco 0464), 1mg Naphthol AS-MX phosphate (Sigma N-4875), 5mg
Fast-blue BB salt (Sigma F-0250) dissolved in 5 mL of distilled water. Alkaline dye mixture solution was added into plates for 30 min protected from light. All images were captured at the same magnification and light intensity.

Alizarin red staining

Human osteoblasts were seeded in 24-well plates at a density of 50,000 cells per well in osteogenic differentiation medium. At 14 days, cells were fixed in 96% ethanol for 15 min and stained with 0.1% alizarin red solution (Alizarin Red S, Sigma), pH 4.2 for 1 h at room temperature as previously described [27, 30]. All images were captured at the same magnification and light intensity. For quantity analysis, the nodules were dissolved by 10% hexadecylpyridinium chloride for 1 h and colorimetric assay was measured at 562nm.

Real-time PCR for osteoblast differentiation markers

For real-time PCR experiments, 50,000 osteoblasts were seeded with PRP or i-PRF in 24 well plates. After 3 and 14 days of culture, total RNA was isolated from osteoblasts cells using AxyPrep™ Multisource Total RNA Miniprep Kit (AXYGEN, Union City, California, USA) according to the manufacturer's protocol. The RNA concentration was determined by a NanoDrop 2000 UV-Vis Spectrophotometer as previously described [31]. A total of 1 ug RNA solution were immediately reverse transcribed to cDNA using a First Strand cDNA Synthesis Kit (GeneCopoeia, Maryland, USA) and the final volume is 100ul. The sequences of primers for collagen type I alpha1 (COL1A), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), osteocalcin (OCN) and GAPDH genes of human are listed in Table I. Real-time RT-PCR was performed using 20ul final reaction volume of All-in-One™ qPCR Mix Kit (GeneCopoeia, Maryland, USA) and the target gene expression was assayed on a CFX Connect™ Real-Time PCR Detection System. The delta-delta Ct method was used to calculate gene expression levels relative to house-keeping gene GAPDH and normalized to control cells. Data were log-transformed prior to analysis by one-way ANOVA with Bonferroni test using Graphpad Software v. 6 (Graphpad Software, La Jolla, CA, USA). All samples were analyzed in triplicate.

Osteocalcin staining

Osteoblasts were plated in 24-well plate at a density of 50'000 cells per well. At two weeks, cells were fixed with 4% formaldehyde for 15 min and permeabilized with 0.5% Triton X-100 (Merck, Germany) for 3 min at room temperature (RT). Subsequently, cells were washed with PBS and incubated with primary antibodies osteocalcin (Santa Cruz Biotechnology Inc., USA) diluted 1:100 in PBS containing 2% bovine serum albumin (BSA, Roche) for 1 h at 37°C. After washing with PBS, cells were incubated with FITC-conjugated-goat-anti-rabbit (Invitrogen) diluted 1:200 in PBS for 1 h in dark conditions at 37°C. The cells were washed again and incubated with DAPI for 3 min. Images were captured from each surface on an Olympus DP71 fluorescence microscope.

Statistical analysis

Statistical analysis was performed by one-way ANOVA with Bonferroni test, using Graphpad Software v. 6 (Graphpad Software, La Jolla, CA, USA) and statistical significance was considered at p < 0.05. All data are expressed as the mean±SE.

Results

Osteoblast viability

As presented in Figure 1, the influence of PRP and i-PRF was investigated on cell viability of human osteoblasts. It was found that more than 95% of the cells remained alive in each group following culture with either PRP or i-PRF with no significant difference among the three groups (Figure 1). It may be concluded therefore that the PRP and i-PRF displayed excellent cell viability and biocompatibility under the present in vitro culture system.

Osteoblast migration, adhesion and proliferation

It was then observed that both PRP and i-PRF promoted cell migration at 24 h. PRP induced a 200% increase in ALP mRNA level when compared to controls whereas i-PRF induced approximately a 280% significant increase when compared to all other groups (Figure 2). The results from the cell adhesion assay showed no significant difference among all three groups at all time points including 2, 4 and 8 h (Figure 3A). The cell proliferation assay revealed that both PRP and i-PRF increased the proliferation rates of human osteoblasts at three and five days when compared to control tissue culture plastic (Figure 3B). Furthermore, i-PRF was significantly higher than PRP at five days (Figure 3B).

Osteoblast differentiation

Lastly, to evaluate the effects of PRP and i-PRF on osteoblast differentiation, human osteoblasts were cultured with ODM for 7 and 14 days and then stained for ALP and Alizarin S Red (Figure 4). At seven days, both PRP and i-PRF demonstrated significantly higher ALP activity when compared to control tissue culture plastic with results demonstrating the significantly highest values in the i-PRF group (Figure 4A, B). The same trend was observed for Alizarin S Red staining. Furthermore, both PRP and i-PRF promoted mineralized nodule formation compared to the control tissue culture plastic, with the staining intensity in the i-PRF group being most noticeable (Figure 4C). The quantified analysis of Alizarin S Red staining confirmed these findings (Figure 4D).

Thereafter, mRNA levels of osteoblast differentiation markers were evaluated by real-time PCR (Figure 5). It was found that while i-PRF provoked a slight significant increase in ALP mRNA level at three days, over a 2 fold increase was observed at 14 days in the i-PRF group when compared to PRP or control tissue culture plastic groups (Figure 5A). Additionally, both PRP and i-PRF increased the expression of OCN, Runx2 and COL1A at 14 days when compared to controls with i-PRF demonstrating the highest expression for genes encoding OCN and Runx2 (Figure 5B, C, D).

Finally, immunofluorescence staining was used to confirm the effect of PRP and i-PRF on osteocalcin expression of human osteoblasts. As demonstrated in Figure 6, PRP exhibited a slight increase in immunofluorescent staining of osteocalcin when compared to the control group, while i-PRF showed significantly higher staining intensity when compared to all other groups. Taken together, the results indicated that both PRP and i-PRF promoted osteogenic differentiation whereby i-PRF had a more significant influence.

Table I. Primer pairs used in the qRT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence</th>
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<tr>
<td>hALPL-F</td>
<td>CCAAGGACGCTGGGAAATCT</td>
</tr>
<tr>
<td>hALPL-R</td>
<td>TATGCACTAGCTGTTAGGCG</td>
</tr>
<tr>
<td>hOCN-F</td>
<td>CTCACACTCCTGCGCCTAT</td>
</tr>
<tr>
<td>hOCN-R</td>
<td>GGTCCTTCTACATCCCTCCTG</td>
</tr>
<tr>
<td>hhRUNX2-F</td>
<td>GCAGCTCATTCTCATCCAGTA</td>
</tr>
<tr>
<td>hhRUNX2-R</td>
<td>GGTCCTAGGTTAGGAGGTTA</td>
</tr>
<tr>
<td>COL1A1-F</td>
<td>TCAGCATGCTGCCAGTGGAC</td>
</tr>
<tr>
<td>COL1A1-R</td>
<td>TCTGTACCCACGGTATGGTG</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>GCACCGTCAAGGCTGAGAAC</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TGGTGAAGACGCGACGAG</td>
</tr>
</tbody>
</table>
Figure 1. Live/Dead assay at 24 h of human osteoblasts treated with PRP or i-PRF (A) The merged fluorescent images of Live/Dead staining with viable cells appearing in green and dead cells in red. (Scale bars=100 μm) (B) Cell viability was quantified as the percentage of living cells in each group. No significant differences was observed among the three groups.

Figure 2. Effect of PRP and i-PRF on the migration of human osteoblasts (A) Cell migration was assessed after 24 h. (Scale bars=100 μm). (B) Normalized cell migration demonstrated a significant increase in cell recruitment number for PRP with significantly elevated levels in the i-PRF groups. (* denotes significant difference between two groups p<0.05, ** denotes significantly higher than all other treatment groups p<0.05)
Figure 3. Effects of PRP and i-PRF on the adhesion and proliferation of human osteoblasts (A) cell adhesion at 2, 4 and 8 h and (B) cell proliferation at 1, 3 and 5 days. (* denotes significant difference between two groups p<0.05, ** denotes significantly higher than all other treatment groups p<0.05)

Figure 4. Effects of PRP and i-PRF on the differentiation and mineralization of human osteoblasts (A) Human osteoblasts were stained for ALP staining at seven days. (B) ALP activities were measured by densitometry at 405nm. (C) Human osteoblasts were stained for Alizarin S Red staining at 14 days. (D) Alizarin S Red staining was quantified by densitometry at 562nm. (* denotes significant difference between two groups p<0.05, ** denotes significantly higher than all other treatment groups p<0.05)

Figure 5. Real-time PCR of human osteoblasts treated with PRP or i-PRF at 3 and 14 days for mRNA levels of (A) ALP, (B) OCN, (C) Runx2 and (D) COL1. (* denotes significant difference between two groups p<0.05, ** denotes significantly higher than all other treatment groups p<0.05)
Figure 6. Immunofluorescent osteocalcin (OCN) staining of human osteoblasts treated with PRP and i-PRF at 14 days. (A) The merged fluorescent images of OCN staining (green) with DAPI staining (blue). (Scale bars=100μm) (B) Quantified values of OCN staining in comparison with control samples (* denotes significant difference between two groups p<0.05, ** denotes significantly higher than all other treatment groups p < 0.05).

Discussion

The aim of the present study was to compare the influence of both PRP and i-PRF on osteoblast migration, proliferation and differentiation. While PRP has now been investigated in numerous studies as a regenerative blood concentrate, more recently the development of newer platelet formulations with the aim of removing secondary by-products such as anti-coagulants has been proposed. Much research has shown that PRP releases the majority of its growth factors at very early time periods whereas PRF induces a more gradual and sustained release of growth factors [32]. It has been shown that within the first 10 min, up to 70% of growth factors are released to the surrounding environment with nearly 100% released within the first hour [32]. These growth factors, despite being released at early time points, have a significant influence on the cellular behavior of many cell types [33–35]. With respect to osteoblasts, many studies have previously shown that PRP has a positive influence on either osteoblast proliferation or differentiation [34,36–38].

Nevertheless, the translational potential of PRP to clinics has seen various results [39–42]. While PRP has been shown to possess advantages on osteoblast differentiation in vitro, it has been hypothesized that the added addition of anti-coagulants has potentially either caused a cytotoxic effect in vivo or significantly reduced the natural coagulation cascade thereby preventing natural wound healing [43–44]. For these reasons, recent groups utilizing anticoagulants have thus suggested their removal from their standardized platelet concentrates that have been utilized for over 15 years [43]. Therefore and for these reasons, an increasing trend of utilizing PRF as a replacement blood-derived growth factor for tissue regeneration has been more heavily utilized most noticeably in the dental field [15]. It has been reported that PRF acts to release growth factors much more slowly as a result of both the use of a fibrin scaffold capable of entrapping growth factors and releasing them slowly over time, as well as additionally housing of leukocytes, a cell-type responsible for additional growth factor release [45]. Interestingly, two recent articles have further shown that by decreasing centrifugation speeds, a higher proportion of leukocytes could be maintained in the upper layer where PRF is collected and thereby increasing total growth factor release [26,46].

In the present study, the low speed centrifugation concept was utilized to produce a roughly 1mL layer of liquid PRF by centrifugation at 700 rpm (only 60G) for 3 min as opposed to conventional PRF centrifugation protocols of 2700 rpm for 12 min. Therefore, while this modified centrifugation protocol produces much less liquid as per not having substantial centrifugation time/speed to separate the two layers, it produces a 1 mL layer of i-PRF that may be utilized in a liquid formulation prior to fibrin clot formation (coagulation) without having to use anti-coagulants; thereby being 100% naturally-derived. Therefore, the aim of this study was to first investigate the effect of PRP and i-PRF on human osteoblast behavior. To the best of these authors knowledge, this is the first cell study evaluating the potential of i-PRF on any cell-type.

In a first experiment investigating osteoblast viability, it was observed that both PRP and i-PRF had excellent biocompatibility without inducing any cell apoptosis/cell death (Figure 1). Furthermore, it was found that both PRP and i-PRF induced a significant increase in cell migration and proliferation whereby the i-PRF group further significantly increased the migration of cells when compared to PRP (Figure 2, 3). Lastly, it was also found that specifically and most noticeably, i-PRF promoted more osteogenic differentiation when compared to PRP and control tissue culture plastic (Figure 4,5,6).

In summary, the present study concludes that this novel formulation of platelet concentrate without the use of anti-coagulants (i-PRF) was able to more remarkably influence osteoblast behavior by influencing the migration, proliferation and differentiation of human osteoblasts when compared to PRP. It may therefore be hypothesized that one of the main reasons for the added benefit of i-PRF is the additional incorporation of leukocytes as well as fibrin proteins that have yet to coagulate. Future research investigating the individual properties of i-PRF by inducing the knockout of either fibrin or by inhibiting leukocyte activity may further determine the role of each component of i-PRF and their specific activity on cellular differentiation. Furthermore, as PRP is presently utilized for an array of clinical procedures, further research comparing PRP to i-PRF across many fields of medicine is necessary in pre-clinical animal research prior to its potential clinical use. Therefore, further animal and thereafter
clinical studies are needed to fully confirm the potential of i-PRF as a new blood platelet concentrate for potential regeneration of tissues.

Acknowledgments

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Declaration of interests

All other authors declare no conflict of interest.

References