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Biological characterization of an injectable platelet-rich fibrin mixture consisting of autologous albumin gel and liquid platelet-rich fibrin (Alb-PRF)

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Abstract
Platelet-rich fibrin (PRF) has been proposed as an autologous membrane with the advantages of host accumulation of platelets and leukocytes with entrapment of growth factors. However, limitations include its faster resorption properties (~2 weeks). Interestingly, recent studies have demonstrated that by heating a liquid platelet-poor plasma (PPP) layer, the resorption properties of heated albumin (albumin gel) can be extended from 2 weeks to greater than 4 months (e-PRF). The aim of the present study was to characterize the biological properties of this novel regenerative modality. Whole blood collected from peripheral blood in 9-mL plastic tubes was centrifuged at 700 g for 8 minutes. Thereafter, the platelet-poor plasma layer was heated at 75°C for 10 minutes to create denatured albumin (albumin gel). The remaining cells and growth factor found within the Buffy coat layer (liquid PRF) were thereafter mixed back together with the cooled albumin gel to form Alb-PRF. Histological analysis, including the distribution of cells within Alb-PRF, was then performed. Seven different growth factor release kinetics from Alb-PRF were characterized up to 10 days, including PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, IGF and EGF. Thereafter, gingival fibroblast cell responses to Alb-PRF were investigated by means of a live/dead assay at 24 hours; migration assay at 24 hours; proliferation assay at 1, 3 and 5 days; real-time PCR for the expression of TGF-β and collagen 1α2 at 3 and 7 days; and collagen 1 immunostaining at 14 days. It was first observed histologically that viable cells were evenly distributed throughout the Alb-PRF formulation. Growth factor release demonstrated a slow and gradual release, particularly for TGF-β1 and PDGF-AA/AB, during the entire 10-day period. Alb-PRF also exhibited statistically significantly higher cell biocompatibility at 24 hours and statistically significantly induced greater fibroblast proliferation at 5 days when compared to those of control TCP. Alb-PRF further induced statistically significantly greater mRNA levels of TGF-β at 3 and 7 days, as well as collagen 1 at 7 days. The present results indicate that Alb-PRF possesses regenerative properties induced by the slow and gradual release of growth factors found in liquid PRF via albumin gel degradation. Future studies are thus warranted to fully characterize the degradation properties of Alb-PRF in vivo and explore future clinical applications in various fields of medicine.

Introduction
A number of different surgical techniques, biomaterials and growth factors have been utilized in regenerative medicine and dentistry to speed the formation of either hard or soft tissues [1–4]. While much advancement has been made with respect to the use of biological agents as key mediators of tissue regeneration, some disadvantages have also been reported, including their associated high costs and potential risk of inflammation [5–7]. Nevertheless, biological agents such as recombinant human platelet-derived growth factor (rhPDGF) and bone morphogenetic protein-2 (rhBMP2) play pivotal roles in modern medicine for the regeneration of complex defects. The use of autologous platelet concentrates has also gained tremendous momentum in recent years as a low-cost regenerative modality with the ability to stimulate tissue neoangiogenesis [4,8,9]. Platelet-rich plasma (PRP) was a first-generation platelet concentrate that favored up to a 6-8-fold increase in platelet concentrations [10–12]. Early experiments revealed the ability of several key growth factors found in blood, including PDGF, transforming growth factor beta 1 (TGF-β1), and vascular endothelial growth factor (VEGF), to be found at higher concentrations when compared to those of whole blood, thus favoring modulation of tissue repair and wound healing [13–18].

More recently, platelet-rich fibrin (PRF) was proposed as a platelet concentrate derived from whole blood without the use of anticoagulants [19,20]. PRF involves the formation of a fibrin clot following centrifugation and may be utilized as a regenerative agent for various surgical, dental and medical applications in various fields of medicine.
with specific concentrations of host platelets and leukocytes as well as autologous growth factors. Furthermore, modifications to protocols as well as centrifugation tubes have further allowed for a working liquid-PRF that may be utilized as an injectable modality similar to PRP[21].

One of the main limitations of PRF has been its short in vivo turnover rate. In regenerative dentistry, it provides only a limited ability to act as a true ‘barrier membrane’ since its use is associated with a typical 10-14-day resorption period in vivo[22]. An interesting attempt using the heat-compression technique with PRF membranes was introduced by Kawase et al., which aimed to use PRF membranes for guide tissue regeneration (GTR) treatment. The heat compressed PRF was observed for at least 3 weeks postimplantation in vivo, whereas the control PRF membranes were completely resorbed within 2 weeks[22]. Furthermore, in facial esthetics and plastic surgery, to overcome the rapid degradation of plasma and keep volume stabilization, a novel technique was developed whereby platelet-poor plasma (PPP) containing mainly roughly 60% albumin is heated at 75 Celsius for 10 minutes to allow for the denaturation and breaking of many of the weak linkages or bonds (e.g., hydrogen bonds) within its protein molecule[23]. Following this process, the proteins are then restructured in a more densely organized protein structure with extended resorption properties for up to 4–6 months [23–25]. However, despite the longer-lasting heat-treated PRF/PPP, a lower regenerative potential is easily expected, since no cells/growth factors in the heat-treated PRF/PPP are capable of enduring the denaturation process (thermal heating). For these reasons, a process whereby reintroduction of the platelet-rich layer from the buffy coat is mixed back into heated PPP (albumin gel) once cooling occurred (termed Alb-PRF)[23].

In view of previous research to date on the topic[23], currently, no single study has investigated or characterized the biological properties of Alb-PRF, which may represent a potential improvement in PRF-based clinical applications. Therefore, the aim of the present study was to perform histological evaluation including the distribution of cells within Alb-PRF and to characterize the release of seven key growth factors found in blood up to a 10-day period, including PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, epidermal growth factor (EGF) and insulin growth factor 1 (IGF-1). Then, cell biocompatibility, migration potential, proliferation assay and expression of TGF-β1 and collagen 1 were investigated in human gingival fibroblasts.

Materials and Methods

Preparation of an Albumin Gel-platelet-rich Fibrin Mixture (Alb-PRF) (Figure 1)

Blood samples were collected with the informed consent of 6 volunteer donors. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. No ethical approval was required for this study because human samples were not identified, as previously described[21]. Nine milliliters of blood in plastic tubes was centrifuged at 700 g for 8 minutes using an Eppendorf centrifugate 5702 machine (Hamburg, Germany). The upper layer (platelet-poor plasma layer) was collected in 2-ml syringes and heated at 75°C for 10 minutes to create denatured albumin (albumin gel). Following heating, the albumin gel was allowed to cool to room temperature for 10 minutes. Then, liquid PRF including remaining cells and growth factor found within the buffy coat layer was thereafter mixed back together with the cooled albumin gel to form Alb-PRF using a female-female luer lock connector. This combination allowed that both the lower-resorption properties of the albumin gel along with the higher cell content and growth factor content of the liquid PRF layer to be remixed. The injectable Alb-PRF gels were then transferred into 6-well cell culture plates forming a gelated membrane.

Histological sample preparation

The Alb-PRF specimens were trimmed to an 8-mm diameter with a biopsy punch (STIEFEL, Middlesex, UK) and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) on ice and embedded in Polyfreeze tissue freezing medium (Sigma, St. Louis, MO, USA) after sucrose equilibration. The frozen specimens were sectioned into 5-μm-thick slices in the chamber of a cryomicrotome (Hyrax C60, Zeiss, Oberkochen, Germany) with an adhesive film (Cryofilm Type 2C(16UF), Section-Lab, Hiroshima, Japan) using Kawamoto’s film method[26]. The sections were stained with hematoxylin and eosin (H&E; Section-Lab). The images were captured with a digital microscope (VHX-6000, Keyence, Osaka, Japan).

Protein Quantification with ELISA

In order to determine the amount of released growth factors from Alb-PRF, samples in 5 ml of culture media (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) were placed into a cell incubator at 37°C to allow for growth factor release into the culture media. At 15 minutes, 60 minutes, 8 hours, 1 day, 3 days and 10 days, 5 ml of culture media was collected, frozen and replaced with 5 ml of additional culture media. Protein quantification was carried out using ELISA. At the desired time points, PDGF-AA (DY221, range = 15.60–1,000 pg/ml), PDGF-AB (DY222, range = 15.60–1,000 pg/ml), PDGF-BB (DY220, 31.20–2,000 pg/ml), TGF-β1 (DY240, range = 31.20–2,000 pg/ml), VEGF (DY293B, range = 31.20–2,000 pg/ml), EGF (DY236, range = 391–250 pg/ml) and IGF-1 (DY291, range = 31.20–2,000 pg/ml) using Kawamoto’s film method[26]. Absorbance was measured at 450 nm and 540 nm using a microplate reader (TECAN Infinite200 pro, Tecan Group Ltd., Männedorf, Switzerland), and the difference values were used for further calculation. Each condition was measured from 6 participants in duplicate.

Cell Culture

Alb-PRF were incubated in cell culture medium for 3 days at 37°C; thereafter, the conditioned medium was collected and utilized in future experiments. 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 were cultured in a humidified atmosphere at 37°C in growth medium consisting of DMEM (Gibco), 10% fetal bovine serum (FBS; Gibco), and 1% antibiotics (Gibco). Cells were seeded with 20% conditioned medium from Alb-PRF contained within 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. DMEM incubated and collected at the same time as the Alb-PRF conditioned medium was used as a control sample.

Cell Viability

At 24 hours post cell seeding on φ13-mm plastic tissue culture coverslips (Sarstedt, Newton, NC, USA), 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 a fluorescent microscope (Nikon Eclipse E800, Nikon, Tokyo, Japan). Thereafter, cells were expressed as percentages of live versus dead cells following cell culture growth with Alb-PRF.

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Cell Migration Assay

The migration assay was performed using a 24-well plate and polyethylene terephthalate filters with a pore size of 8 μm (ThinCert™, Greiner Bio-One GmhH, Frickenhausen, Germany). After starving the cells in DMEM containing 0.5% FBS for 12 hours, 10,000 resuspended cells were seeded in the upper compartment in DMEM containing 0.5% FBS. The 20% conditioned medium in DMEM containing 10% FBS was filled into the lower compartment of the wells. After 24 hours, cells were fixed with 4% formaldehyde for 2 minutes. Thereafter, cells were permeabilized by methanol (Sigma) for 15 minutes and stained with Giemsa solution (MERCK, Darmstadt, Germany) for 20 minutes. The upper side of the Image 1. Alb-PRF preparation protocol. 1) Whole blood (79 mL) was centrifuged at 700 g for 8 minutes. The upper layer (yellow layer) shows the liquid plasma layer. 2) The most upper layer of platelet-poor plasma (PPP) was collected in a syringe. 3) The collected PPP was heated in a heat block device at 75°C for 10 minutes and thereafter 4) cooled to room temperature for approximately 10 minutes. An injectable albumin gel was then prepared. 5) The liquid platelet-rich layer (liquid-PRF), including the buffy coat layer with accumulated platelets and leukocytes, was collected in a separate syringe. 6) The albumin gel and native liquid PRF were then thoroughly mixed by utilizing a female-female luer lock connector. 7) Injectable Alb-PRF in final ready form.
the filter membrane was 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 by the counting tool of the digital microscope (Keyence).

**Proliferation Assay**

HGF-1 cells were quantified by a luminescent cell viability assay (CellTiter-Glo®, Promega, Madison, WI, USA) at 1, 3 and 5 days. At desired time points, living cells were quantified using a luminescence plate reader (TECAN Infinite200 pro).

**Real-time PCR Analysis**

Total RNA was harvested at 3 and 7 days post-stimulation of HGF-1 cells to investigate the mRNA levels of TGF-β and collagen 1a2 (COL1a2). Primer and probe sequences for genes were fabricated with primer sequences according to Table I. RNA isolation was performed using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland). A Nanodrop 2000 (Thermo, Wilmington, DE, USA) was used to quantify total RNA levels. Real-time RT-PCR was performed using the GoScript™ Reverse Transcription system (Promega) and quantified on an Applied Biosystems 7500 fast machine using GoTag® qPCR Master Mix (Promega). The ΔΔCt method was used to calculate gene expression levels normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**Collagen Immunofluorescent Staining**

At 14 days post seeding on plastic tissue culture coverslips, HGF-1 cells were fixed with 4% formaldehyde for 10 minutes, followed by PBS containing 0.2% Triton X-100 and blocked in PBS containing 1% bovine serum albumin (BSA, Sigma) for 1 hour to permeabilize the cells. Subsequently, the cells were incubated overnight at 4°C with mouse monoclonal collagen type I (COL-1) antibodies (sc-39772, Santa Cruz, Dallas, TX, USA) diluted 1:200 in PBS containing 1% BSA. After washing with PBS, cells were incubated for 1 hour at 37°C with FITC-conjugated mouse IgG binding proteins (sc-516140, Santa Cruz) diluted 1:100 in PBS containing 1% bovine serum albumin (BSA, Sigma). Images were captured by a Nikon Eclipse E800 fluorescence microscope. The optical density of the fluorescent collagen staining was quantified by a Nikon Eclipse E800 fluorescence microscope. DAPI nuclear staining (Vector, Burlingame, CA, USA). Images were captured by a Nikon Eclipse E800 fluorescence microscope. The optical density of the fluorescent collagen staining was quantified by a Nikon Eclipse E800 fluorescence microscope. DAPI nuclear staining (Vector, Burlingame, CA, USA). Images were captured by a Nikon Eclipse E800 fluorescence microscope.

**Statistical Analysis**

Means and standard errors were calculated, and the data were analyzed for statistical significance using an unpaired t-test for the cell viability, migration assay, and collagen staining and two-way analysis of variance for the proliferation assay and real-time PCR analysis with the Bonferroni test (p values < .05 was considered significant) by GraphPad Prism 8.2 software (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**Microscopic Observation of Alb-PRF**

The frozen sections of Alb-PRF were prepared using film transfer methods to avoid the shrinkage of samples. H&E staining of Alb-PRF sections showed two composite structures with denatured liquid PRF (albumin gel) particles and gelated liquid PRF (Figure 2a–c). The loose matrix structure presented gelated liquid PRF, including several leukocytes entrapped within the fibrin fibers (Figure 2d). The eosin-stained dense structures showed albumin gel, namely, denatured plasma components including few leukocytes (Figure 2e).

**Growth Factor Release from Alb-PRF**

The release of growth factors from Alb-PRF was investigated by ELISA, including PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, VEGF, EGF and IGF-1 (Figure 3). Alb-PRF demonstrated a continuing release of growth factors up to 10 days (Figure 3a). The total release of growth factor calculations revealed that TGF-β1 released the highest quantities of growth factors among the 7 tested, followed by PDGF-AA and PDGF-AB (Figure 3b).

**Biocompatibility of Alb-PRF on Human Gingival Fibroblasts**

In the first cell culture experiment, the effects of Alb-PRF were investigated on the cell viability of human gingival fibroblasts (HGF-1 cells). It was found that Alb-PRF demonstrated excellent cell biocompatibility by demonstrating most notably high living cells (green cells, Figure 4a) with very few observable apoptotic cells (red cells). The quantified data suggested that Alb-PRF was fully biocompatible under the present in vitro cell culture model (Figure 4b).

**Influence of Alb-PRF on Human Gingival Fibroblast Activity**

Alb-PRF was then investigated on gingival fibroblasts for the potential of cell migration, proliferation and expression of TGF-β and COL1 (Figure 4c–g). Alb-PRF induced comparable cell migration when compared to that of control media (Figure 4c). Thereafter, a proliferation assay demonstrated that Alb-PRF induced significantly higher cell numbers at 5 days when compared to those of the control (Figure 4d). Investigation into mRNA levels revealed that Alb-PRF was able to significantly increase TGF-β expression at 3 and 7 days postseeding when compared to expression in the control (Figure 4e). Similarly, Alb-PRF significantly increased the mRNA levels of COL1a2 on 7 day (Figure 4f). However, a nonsignificant increase in COL1 immunostaining was observed in the Alb-PRF group on 14 day (Figure 4g).

**Discussion**

The present in vitro study has investigated the regenerative potential of Alb-PRF through histological evaluation, growth factor release, and cell activity. To date, no experimental data exist regarding the combination of liquid PRF mixed with albumin gel. Currently, all known devices have utilized either albumin gel alone, which is completely devoid of cells and growth factors owing to the high processing temperatures, or have investigated liquid PRF, which has more cellular activity but lacks degradation properties past 14 days. By combining both technologies, our group hypothesized that Alb-PRF would demonstrate viable cells capable of releasing growth factors over time while simultaneously improving the cellular activity of gingival fibroblasts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTGFB1 F</td>
<td>ACTACTACGCAAGGAGGTTCA</td>
</tr>
<tr>
<td>hTGFB1 R</td>
<td>TGGTTGGAACCTGGTAGATTTGTCG</td>
</tr>
<tr>
<td>hCOL1a2 F</td>
<td>CCCAGCAGAACAGGTTGATAGG</td>
</tr>
<tr>
<td>hCOL1a2 R</td>
<td>GGCTGGCCAGCTTAGATTTTTC</td>
</tr>
<tr>
<td>hGAPDH F</td>
<td>AGCCCATCGCTGACAC</td>
</tr>
<tr>
<td>hGAPDH R</td>
<td>GCCAATACGACCAATCC</td>
</tr>
</tbody>
</table>

Table I. Primers for quantitative RT-PCR (F = forward primer; R = reverse primer).
Interestingly, to the best of the authors’ knowledge, this is the first study that has investigated Alb-PRF at the cellular level. By heating and denaturing albumin, a modification in the secondary structure after heating transforms the matter into a tridimensional structure. During this heating process, new hydrogen and disulfide ligations in the enzymes are created, which favors a larger tridimensional structure with drastic changes in its resorption properties and improves its stability over time[28]. Results from preclinical animal studies have
shown that while standard PRF resorbs within a 2-week timeframe, denatured albumin lasts up to 4–6 months when implanted into a subcutaneous nude animal model (currently under review). This change in processing adds approximately 20 minutes to the current protocol time to fabricate Alb-PRF, as highlighted in Figure 1; however, the clinical benefits could favor this technology for many clinical indications in medicine and dentistry. It therefore becomes possible to create a true ‘barrier’ or ‘filler’ biomaterial derived from 100% autologous whole blood with drastically extended resorption properties. Furthermore, the ability to harvest large doses from a simple blood draw makes this biomaterial an extremely attractive regenerative agent that may be harvested at low cost from a peripheral vein.

In the present study, it was first observed that by utilizing a standard female-female luer lock connector between two syringes, it was possible to actively infiltrate albumin gel with cells and growth factors from liquid PRF. Figure 2 further demonstrates that the Alb-PRF matrix appeared as a dense scaffold with cells present spanning throughout the autologous biomaterial. This differs drastically from previously utilized albumin gel protocols in that the reintroduction of cells, highly concentrated specifically within the buffy coat layer, is capable of replantation back within the Alb-PRF scaffold. This represents a novel strategy for improving both the clinical performance of either liquid PRF alone or albumin gel alone.

In the present study, it was first observed that when gingival fibroblasts were cultured with Alb-PRF, an actual improvement in growth factor release was experienced from 15 minutes to 10 days (Figure 3). Specifically, TGF-β1 was surprising and drastically increased in concentration, with a nearly 10-fold increase in growth factor release from baseline at 15 minutes up to 10 days. In contrast, other growth factors such as VEGF observed little...
differences/increases in concentration over time. Previous data published by our group have also demonstrated that the previously investigated release of growth factors from PRF membranes and liquid injectable PRF (i-PRF) demonstrated a similar trend, whereby TGF-β1 and PDGF growth factors were released in the highest quantities [21,29,30]. This illustrates that the cells present in Alb-PRF are fully vital and release growth factors in a similar manner to previously published data on standard-based and liquid-based PRF matrices.

The main aim of the present study was to investigate the regenerative potential of Alb-PRF at the cellular level. As such, a gingival fibroblast model was utilized owing to our group’s previous experience utilizing this cell type as well as owing to the ability of PRF to promote soft tissue healing when compared to healing of hard tissues [4,31]. It was first observed that culturing fibroblasts with Alb-PRF actually significantly improved their cellular viability, as confirmed using a live/dead cell assay (Figure 4a). It also promoted cell proliferation at 5 days and induced a significant increase in TGFβ1 and COL1a2 mRNA expression at either 3 or 7 days postseeding. This highlights that unlike traditional biomaterials that may be utilized in dentistry and medicine with extended resorption properties (such as polymers in the facial esthetic field or nonresorbable PTFE membranes in dentistry), this completely autologous biomaterial is not only low cost but also actually promotes the regeneration of cells within its surrounding implanted microenvironment. It also avoids any potential foreign body reaction since it is totally autologous and additionally improves the growth factor release from local cells. Future animal research will be pivotal to further understand the full potential of Alb-PRF in clinical practice.

Not surprisingly, the scientific literature still lacks adequate reports, either in the form of case series or randomized trials, on the real clinical advantages pertaining to the use of Alb-PRF. Since Alb-PRF offers significant structural advantages in its degradation properties, it remains to be investigated if in fact Alb-PRF would release growth factors for even further time periods past 10–14 days owing to its slower degradation rate.

Several clinical applications may also be conceptualized, providing almost immediate impact. For example, it is known that the use of titanium mesh for ridge augmentations in dentistry is associated with a relatively high rate of mesh exposure, with reports up to 50% [32–35]. Currently, many clinicians have utilized PRF overtop of a titanium mesh or overtop of a Ti-mesh +collagen membrane to minimize the rate of exposure. By utilizing a longer-lasting Alb-PRF membrane for such an application, it would theoretically be hypothesized that further improvements would be expected. Future clinical studies are therefore needed. Similarly, many filler-type biomaterials are utilized in facial esthetics. While many patients prefer the natural’ and more autologous use of liquid plasma, the main drawback to date has been the faster than desired resorption rate of liquid PRF with relatively quick facial volume loss. Therefore, by utilizing Alb-PRF, a preferential extended clinical outcome may be expected. Future research is also needed in this field.

In conclusion, the present study revealed for the first time the in vitro positive regenerative properties of Alb-PRF as a low substitution platelet concentrate with extended resorption properties. Alb-PRF may be used as an injectable form utilizing a female-female luer lock connector or with a scaffold or membrane form by pre-forming in custom-sized trays to create specific and desirable shapes. Furthermore, Alb-PRF remains moldable upon either injection or during its shaping with a long-lasting growth factor release curve capable of stimulating tissue regeneration over extended periods of time. Future animal studies and clinical testing are therefore needed to further validate this novel regenerative technology.

Conflict of Interest
Richard Miron declares that he has intellectual property on the Bio-Heat technology utilized in this manuscript. All other authors declare no conflict of interest.

References


