Original Article

DOI: 10.6966/EDMJ.202203_9(1).0001

Investigate the Positive Impact of in Vitro Release of Growth Factors from Platelet-Rich Fibrin Releasates

Shi-Yuan Sheu[1,3], Ming-Hsi Chuang[4,5], Yi-Chih Lei[2,6], Shun-Chieh Yu[1], Yu-Siang Luo[6], Chia-Yih Wang[7,8,], Tzong-Fu Kuo[2,6,*]*

Objective: The aim of the study was to evaluate the various growth factors (GFs) released for a period of 300 minutes (which covers the maximum time needed for surgical or day care procedures) for a feasibility trial from platelet-rich fibrin releasates (PRFr).

Methods: Six healthy donors were recruited and 64 mL of blood sample was withdrawn from each individual. Following the standard centrifugation protocol, platelet-rich fibrin (PRF) and PRFr were prepared. The platelet-derived growth factor-AB (PDGF-AB), insulin-like growth factor-1 (IGF-1), transforming growth factor β-1 (TGF-β1), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), and nerve growth factor (NGF) were evaluated in PRFr over 300 minutes after clot formation.

Results: The comparison between the final released amounts and the initial content in PRFr: PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF and NGF levels were 1.7-, 4.2-, 5.7-, 2.2-, 7.7-, 3.1- and 2.2-fold higher, respectively, in the 300-minute period than in the 5-minute period. The content of GFs in the releasates at 300 minutes were significantly different from respective content at 5 minutes.

Conclusion: The PRFr could be used in day-surgery procedures after formation to maximize release of GFs to the wound site, theoretically enhancing the potential for tissue healing.

Key words: growth factors, platelet-rich fibrin, platelet-rich fibrin releasates, day-surgery procedure

Introduction

There is considerable research interest in using platelet-rich fibrin (PRF) alone or in combination with grafting materials for topical applications in orthopedic procedures,[1,2] periodontal reconstructive surgeries,[3] nerve repairs,[4] etc. This clinical application was endorsed by evidence that several major

From the [1]Department of Integrated Chinese and Western Medicine, Chung Shan Medical University Hospital; [2]Department of Post-Baccalaureate Veterinary Medicine, Asia University, Taichung; [3]Department of Chinese Medicine, E-Da Cancer Hospital, Kaohsiung; [4]Gwo Xi Stem Cell Applied Technology; [5]Department of Bioinformatics, Chung Hua University, Hsinchu; [6]School of Veterinary Medicine, National Taiwan University, Taipei; [7]Department of Cell Biology and Anatomy, College of Medicine and [8]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan.
(* Signifies equal contributions as corresponding authors)
Received: October 30, 2020 Accepted: January 14, 2021
* Address reprint request and correspondence to: Tzong-Fu Kuo, Department of Post-Baccalaureate Veterinary Medicine, Asia University, No.500, Lioufeng Road, Wufeng District, Taichung City 41354, Taiwan.
Tel: +886-4-2332-3456 ext. 1843, E-mail: tzongfu@asia.edu.tw

growth factors (GFs) are contained at high levels in PRF preparations that are known to play a crucial role in tissue repair.[5-7] PRF-GFs exhibit chemotactic and mitogenic activities that promote and modulate cellular functions involved in tissue repair and regeneration, cell migration, and proliferation.[8]

The PRF, a second-generation platelet concentrate introduced by Choukroun et al. in 2001 and has been widely used in the field of oral and maxillofacial surgery, was prepared without the use of anticoagulants and thrombin additives.[9] It is known to provide immune-compatible GFs at a very low cost and has shown a gradual and slow release of platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).[10,11] Standard (Choukroun's) PRF is centrifuged at 3,000 rpm for 10 minutes after which it settles into the following layers: red lower fraction containing red blood cells, upper straw-colored cellular plasma, and the middle fraction containing the rigid and elastic fibrin clot. Rapid activation of the coagulation cascade and synthesis of thrombin takes place during centrifugation, thus inducing fibrin formation and platelet activation.[12] As we previously demonstrated, the specific blood collection tubes with clot activator and gel has been shown to obtain the PRF that we suggested are easier to perform, thus enhancing lab productivity and workflow.[13]

As an autologous fibrin-based membrane, matrix or scaffold containing platelets and leukocyte GFs derived from human blood, PRF is composed of a dense, stable fibrin network via interactions between the alpha(IIb)beta(3) integrin and the fibrin network that allows slower release of GFs compared to platelet-rich plasma (PRP).[14] This is in contrast to PRP, which is activated via concentrated thrombin conversion making a rapid polymerization, followed by strong contraction of the clots, from which fluids will expel; the process may cause difficulties for PRP in entrapping cytokines released from platelets.[15] Thus, PRP releases significantly more GFs when compared to PRF during the first 15 – 60 minutes after clot formation, while PRF displays a continual and steady release of a modest amount of growth factors over a 10-day period.[16] Recent studies further confirmed the gradual release of PDGF and TGF for 28 days from PRF formation, whereas they are released within one day from PRPs.[17] In this article, we used human PRF gels obtained from centrifuged whole blood (tubes with a clot activator such as BD Vacutainer® as previously mentioned)[1,13,18,19] to access the various GFs content in PRF releasates and time course over 300 minutes after clot formation, in an attempt to develop a better understanding of the content and to suggest improved procedures for better therapeutic applications.

Materials and Methods

Study design and ethics statement

The research was conducted in Chung Shan Medical University Hospital. Ethical clearance was obtained from the institution (CSMUH No.CS2-19104). Six healthy young adult donors in this study (four males and two females, non-smokers) were aged 21 – 42 without systemic diseases or under any medical treatments. Informed consent was obtained from the participants fulfilling the criteria for enrolment in the trial. For each individual, eight (8 mL each) tubes of peripheral blood were collected.

Platelet-rich fibrin preparation

The PRF was prepared using the method described in the authors' earlier feasibility studies.[1] Briefly, 8 mL of blood was collected from volunteers in BD Vacutainer® SSTTM collection tubes (cat. #367988) with clot activator and gel (Becton, Dickinson and Company,

New Jersey, USA). The vacutainer was rotated gently for 30 seconds and immediately centrifuged at 3,000 rpm for 10 minutes using a DSC-200A-2 table top centrifuge (Digisystem, Laboratory Instruments Inc., New Taipei City, Taiwan). The resultant jelly-like PRF needed to be separated from the gel-clot without the red blood cells sinking to the bottom of the tube. Forceps were used removed the freshly-generated PRF gels and kept in sterile vials. All preparation and delivery steps were carried out under standard disinfection procedures.

Platelet-rich fibrin releasates preparation

Each of the six PRF samples were collected, divided into four equal segments, transported to four different tubes, and allowed to stand quiescence for 5, 60, 120, and 300 minutes, respectively, to allow PRFr formation. Following centrifugation of the PRF gels at 5,000 rpm for 10 minutes, the supernatant PRFr were carefully aspirated and frozen at -80°C till GFs measurements.

Growth factors assays

Each GF was quantitively measured using sandwich-type ELISA kits as follows: human PDGF-AB (ab100623, Abcam, Cambridge, UK), human IGF-1 (ab100545), human TGF-β1 (ab100647), human VEGF (ab100662), human EGF (ab100504), human b-FGF (ab99979), and human NGF (ab99986). The detection limit was 10.24, 0.1, 0.082, 8.23, 0.82, 102.4, and 6.86 pg/mL for PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF, and NGF, respectively. All steps were performed according to manufacturer's instructions. Standards and samples were assayed in duplicate for each GF and mean values were calculated. Results were obtained by first measuring absorbance at 450 nm then determining GF concentration against a standard curve. We determined the amounts of GFs released from each PRF were multiplied by the total volume of samples.

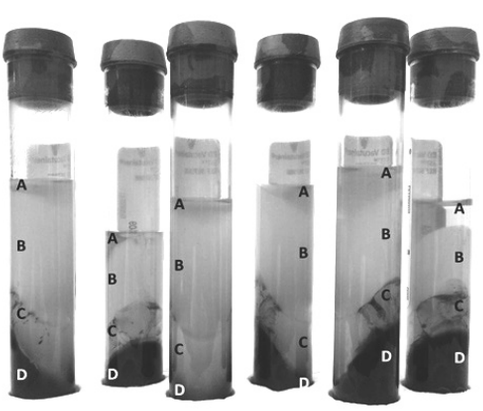

Fig. 1 Venous blood sample following centrifugation indicating the PRF clot in the middle layer of the BD#367988 tube. (A) supernatant serum; (B) platelet-rich fibrin (PRF); (C) clot activator and separation gel; (D) coagulated red blood cells.

Statistical analysis

Results are presented as mean ± standard deviation. The release of seven GFs from the PRF was statistically evaluated using SPSS software v.10.0 (IBM Corp., Armonk, NY, USA). Statistical comparison was evaluated using independent sample t-test. A value of $p < 0.05$ was considered as statistically significant.

Results

A total of 6 participants met the enrollment criteria and provided blood samples. Figure 1 shows a typical human PRF clot harvested after whole blood centrifugation. The mean total content of PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF, and NGF in PRFr at different time points over a 300-minute period is shown in Table 1. Significant amounts of each GF were found over the time course in this study. On average, content in the releasates were 69.6 ± 23.2, 31.5 ± 4.8, 15.9 ± 3.3, 3.7 ± 0.6, 0.7 ± 0.3, 0.31 ± 0.13, and 0.16 ± 0.04 ng at 300 minutes for PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF, and NGF respectively, significantly different from respective content at 5 minutes ($p < 0.05$). By contrast, PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF and NGF

Table 1. Quantity of GFs (PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF, and NGF) in PRFr during each experimental period (n = 6).

Time (min)	Amounts of released GFs (mean ± standard deviation, ng)						
	PDGF-AB	IGF-1	TGF-β1	VEGF	EGF	b-FGF	NGF
5 – 60	41.8 (± 15.1)	7.6 (± 1.3)	2.8 (± 1.0)	1.7 (± 0.4)	0.09 (± 0.03)	0.10 (± 0.03)	0.06 (± 0.01)
60 – 120	55.0 (± 16.7)	18.0* (± 2.7)	6.1* (± 2.1)	2.6 (± 0.4)	0.26* (± 0.11)	0.13 (± 0.04)	0.08 (± 0.01)
120 – 300	64.1* (± 21.0)	24.4* (± 4.1)	10.0* (± 2.9)	3.0* (± 0.5)	0.44* (± 0.19)	0.20* (± 0.07)	0.12* (± 0.03)
> 300	69.6* (± 23.2)	31.5* (± 4.8)	15.9* (± 3.3)	3.7* (± 0.6)	0.70* (± 0.30)	0.31* (± 0.13)	0.16* (± 0.04)

*$p < 0.05$ compared to 5 min.

levels were 1.7-, 4.2-, 5.7-, 2.2-, 7.7-, 3.1- and 2.2-fold higher, respectively, in the 300-minute period than in the 5-minute period. This indicates that GFs were concentrated in the fibrin network of the PRF clot when fibrin polymerization took place, which is the main entity currently used in regenerative medicine.

Discussion

Platelet concentrates (PCs) are autologous biologics derived from whole blood that are preferentially consisting of supraphysiological concentrations of platelets and GFs. The two emerging PCs are PRP and PRF which are basically fibrin matrices enmeshed with morphogenic proteins (GFs) and leukocytes. According to Mosesson et al.[20] who described the structural and biological features of fibrinogen and fibrin in detail, the three-dimensional organization of fibrous networks depends on an activation pathway: In PRP, bilateral junctions are constituted with strong thrombin concentrations and allow the thickening of fibrin polymers. This leads to the constitution of a rigid network, which is not very favorable to cytokine enmeshment and cellular migration.[21] In PRF, weak concentration of thrombin leads to equilateral junctions which result in a fine and flexible fibrin matrix that enable cytokines enmeshment and cellular migration. Moreover, this organization results in the formation of a flexible, elastic, and very strong PRF matrix, thereby providing mechanical support and serving as biologic connectors.[22] Unlike PRP, PRF results from a natural and progressive polymerization that occurs during the centrifugation process, a simplified processing technique not requiring biochemical blood handling, thus makes it superior to PRP. The presence of the leucocytes in the fibrin network and the slow release of cytokines and GFs results in the self-regulation of inflammatory and infectious process and the continued process of healing. The application of PRF offers several advantages including promoting wound healing, bone growth, maturation, graft stabilization, wound sealing, hemostasis, and improving the handling properties of graft materials.[23]

GFs are potent wound healing promoters which accelerate incisional wound repair by different mechanisms. TGF-β1, as a cellular stimulator, increases synthesis of extracellular matrix and stimulates granulation, tissue modulating, and infiltrated immune cells.[24] PDGF, as a chemotactic agent for inflammatory cells with mitogenic activity, activates monocytes and stimulates collagen production.[24] VEGF, a key growth promoter of angiogenesis, supports and coordinates the development of initial cicatricial structures such as vascular tubes.[11] EGF is a common mitogenic factor that stimulates the proliferation of different types of cells, especially fibroblasts and epithelial cells.

In addition, EGF intervention can positively impact both mesenchymal and epithelial cells, reducing inflammation and stimulating the recruitment of precursor circulating cells that promote the formation of new blood vessels.[25] IGF-1 plays an essential role in metabolic pathways and glucose metabolism, coordinating the response to nutrient intake and initiating the appropriate metabolic changes that enable cells to tolerate a variety of stressful stimuli and resist apoptosis as well as initiate the tissue repair response that occurs after injury.[26] b-FGF is a potent angiogenic factor and endothelial cell mitogen and may be an important positive regulator of leukocyte recruitment in acute and chronic inflammation.[27] NGF plays a vital role in reconstructing peripheral nerve fibers and promoting axonal regeneration.[28] As in the Evanson et al. study demonstrated that both age and sex influence the levels of GFs in PRPs,[29] and this effect could account for inconsistencies between reports of the clinical benefits of platelet treatment. Thus, the diverse action of the GFs forms a key player in wound healing and tissue regeneration whereas age and sex should be considered in any PRF investigation.

The aim of the present study was to quantitate the release of various GFs contained in PRFr at 5-, 60-, 120-, and 300-minute intervals. Although this study was designed with a small sample size, there could be genetic, hormonal, or environmental reasons for sex- and age-related differences in GFs levels that could not be fully eliminated in this pilot trial. Blood samples were obtained and collected into BD Vacutainer with clot activator and gel for separation (Ref. 367988). The data showed an increasing content of PDGF-AB, IGF-1, TGF-β1, VEGF, EGF, b-FGF, and NGF in the PRFr over the time course of the study to a total content of about 69, 31, 15,3, 0.7, 0.3 and 0.1 ng, respectively. All the GF exhibited similar release profiles, characterized by a constant increase of the release during the first 60 minutes, with a significantly larger release within 120 to 300 minutes. The total quantity of released factors was in all cases significantly higher than the total amounts extracted just after clot preparation. These results were consistent with the finding where the release profile of the GFs (with the exception of IGF-1) within 300 minutes were observed,[12] an abundant release seen in PDGF-AB, IGF-1, TGF-β1 and VEGF while EGF, b-FGF and NGF have been showing a gradual increase. Some studies have shown that the concentration of GFs varies according to the production protocol of PRF,[12,14] mainly related to the g-force and the type of collection tubes used. Our results, derived in respect to PRFr, demonstrated a constant and gradual increase in the release of GFs. This could be attributed to a stronger fibrin architecture entrapping a greater number of leukocytes in the fibrin matrix, which allows an intense slow release of growth factor from the fibrinogen and fibrin matrix.[30] Dohan et al. also proved a slower release of growth factors from PRF than PRP and observed better healing properties with PRF.[5,10,11,22]

Since the fibrin matrix is better organized, it is able to support cytokine enmeshment and more efficiently direct cell distribution, cellular migration, and the healing program.[23] Our earlier work demonstrated the benefits of implantation of PRF and cartilage granules in the rabbit knee to promote cartilage repair, which resulted in promising short-term results.[2] We further reported the use of a porcine model to evaluate cartilage repair in vivo and evaluated the feasibility of PRF as matrix in a combination of cartilage fragments as a cell source to develop a new autologous chondrocyte implantation (ACI) approach.[1] Another study was conducted with the dental bud cells (DBCs) seeded into PRF to demonstrate the concept that DBC fibrin glue PRF can regenerate a complete tooth in the porcine jaw.[3] More investigations of the effectiveness of using the in vitro release of GFs from PRFr (and then let stand for at least 5 hours) to support the therapeutic appli-

cation in exploratory studies are being conducted. In combination with PRFr, adipose-derived mesenchymal stem cells (ADSCs) can improve healing of an acute osteochondral defect in vivo in the first 14 weeks postoperatively.[18] The PRFr exhibits significant growth factor activity so that it can be administered with ADSCs or chondrogenic mesenchymal stem cells (MSCs) to damaged tissues by either transplanting cells or, as a safer alternative, using the conditioned medium of ADSCs. Recent research has shown that the transplantation of PRFr combined with ADSCs might provide an alternative strategy for the treatment of various bone disorders in osteoporosis with an unlimited source of cells and releasates.[19] Additionally, it is significant that the combined strategy of PRFr with ADSCs decreases axonal and myelin damage after sciatic nerve injury in rats and appears to have a positive effect on the recovery of walking function after injury, thus paving the way for nerve damage repair, speeding up the regeneration of nerves and improving recovery quality.[13] Comprehensively, GFs recruit stem/progenitor cells from their perivascular or other niches to replace the damaged cells by differentiating into a specific cell phenotype and proliferation in the area of injury. All these aspects of the combined approach of PRFr + ADSCs may offer a promising option for repairing damaged cells or tissues to restore normal function.

Conclusion

This study provides recommendation on the release profile of GFs from PRFr that could be used within day-surgery procedures after PRF formation to maximize release of GFs to the wound site.

Acknowledgement

This study was support in part by the following grant number: Chung Shan Medical University Hospital (CSH-2020-A-029) and Gwo Xi Stem Cell Applied Technology Co., Ltd, Taiwan.

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