A Novel Injectable Gel Incorporating Human Platelet Rich Plasma (PRP) Lysate for Periodontal Regeneration: In Vitro Studies

A novel injectable gel incorporating human platelet rich plasma (PRP) lysate for periodontal regeneration: In Vitro Studies

A Thesis Presented by:

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To

The Faculty of Medicine

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Medical Sciences

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Harvard School of Medicine; Massachusetts Institute of Technology; Veterans Affairs Boston Health Care System

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Boston, Massachusetts

April 2019

HARVARD UNIVERSITY

Harvard School of Dental Medicine

DISSERTATION ACCEPTANCE CERTIFICATE

The undersigned, appointed by the Harvard School of Dental Medicine Office of Research have examined a dissertation entitled,

"A novel injectable gel incorporating human platelet rich plasma (PRP) lysate for periodontal regeneration: In Vitro Studies".

presented by **Livia R. P. Valverde**, candidate for the degree Doctor of Medical Sciences hereby certify that it is worthy of acceptance.

Signature: _____

Typed Name: Dr. T. Howard Howell

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Typed Name: Dr. German Gallucci

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Typed Name: Dr. Bernard Friedland

April 30th, 2019

Dedication

To my parents, who have always been an inspiration to me. Thank you for all your love, support and encouragement to pursue my dreams.

To my best friend and husband, Guilherme, for your love, patience and for pushing me forward even when I thought I could not go any further.

And to my son, Lucas, for being the sunshine of my life.

I love our family to the moon and back!

You made it all worth it!

Acknowledgments

I would like to express my sincere gratitude to my advisor and mentor, Dr. Myron Spector for the opportunity of doing research in the bioengineering field. His guidance and support added to his immense knowledge were fundamental for the development and completion of this research project.

Also, I would like to thank Dr. Giuseppe Intini for co-mentoring this project. I have learned so much from you! You're beyond being a great mentor. You were always there for the residents, giving us the much-needed emotional support throughout our residency. So, thank you for being there for us!

I would like to thank my colleagues at Dr. Spector's lab for their support at all times, specially Wanting Niu for her patience and willingness to teach and help me throughout the experiments. My appreciation as well to all other lab colleagues that I've met along the way. Also, I would like to thank Zahra Aldawood from Dr. Intini's lab for sharing her experience in lab with me. I wish we could have worked together more! Thank you for all your help and friendship.

My greatest gratitude to my Program Director, Dr. David Kim, for his support and countless opportunities he gave me throughout the Program.

To Dr. Howell, for since the beginning having supported me and guided me to achieve my academic goals. Your influence in my life is beyond the academic life. Thank you for your mentorship and professional wisdom. You are my role model.

Thank you to all my professors who directly or indirectly helped me to advance in my profession throughout these years. As quoted by Henry Adams: "A teacher affects eternity; he can never tell where his influence stops".

I would also like to thank the members of my Oral Qualifying Committee Dr. Shigemi Nagai, Dr. Yefu Li and Dr. Bruce Paster, Thesis Proposal Committee Dr. Shigemi Nagai, Dr. Yefu Li and Dr. T. Howard Howell, and Thesis defense committee Dr. German Gallucci, Dr. Bernard Friedland and Dr. T. Howard Howell, for their time, insightful comments, and valuable advices on my pathway towards my degree.

My heartfelt thanks to my family for their endless love, support and encouragement throughout my life and studies. Thank you for believing in me!

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ABSTRACT

Background: Platelet rich plasma (PRP) has been utilized as an adjunct to various periodontal procedures with the goal of potentializing the regenerative outcome of surgical therapy. The biopolymer gelatin hydroxylphenylpropionic acid (Gtn-HPA) has recently been developed and proposed to be used as an injectable scaffold for tissue engineering. The aim of the present study was to characterize the influence of the Gtn-HPA hydrogel incorporating PRP on osteoprogenitor cells behavior and growth factor release in vitro.

Methods: Platelet rich plasma was obtained from 6 human donors followed by platelet counting. The mRNA levels of osteoblast differentiation markers including OCN and COL1A1 plus the growth factors receptors FGFr, EGFr and PDGFr were analyzed in 2D cultures, hydrogel only, 10% PRP, 20% PRP, 10% FBS and 20% FBS in a 2% Gtn-HPA hydrogel (n=3) and seeded with osteoprogenitor cells. Furthermore, protein quantification for PDGF, TGF-B, EGF, FGF and IL-1B (n=5) were analyzed in the PRP lysate samples.

Results: Platelet concentration increased between 5 to 9-fold in PRP compared to whole blood counts. Protein quantification was consistently greater for TGFB, PDGF and EGF. Gene expression analysis showed a tendency of upregulation of the osteoblast differentiation markers and growth factor receptor genes in the PRP groups, however a great variability between the donors was noticed.

Conclusion: Within the limitations of this study, the use of human PRP in a Gtn-HPA hydrogel reveals this biopolymer as a promising and suitable carrier/scaffold with optimal growth factor release and enhanced gene expression favoring tissue regeneration.

Keywords: platelet rich plasma, regeneration, hydrogels

INTRODUCTION

It is well established that oral biofilm accumulation is the main etiologic factor for periodontal diseases (1, 2). The pathological process starts when there is a microbial succession from healthy oral microflora to periodontopathogens, mostly anaerobic and gram-negative bacteria, leading to inflammation of the periodontal tissues (3, 4). If left untreated, this infectious disease causes loss of the supporting tissues creating infrabony defects and ultimately tooth loss (5). Therefore, periodontal regeneration has been widely investigated and the desired treatment option in order to overcome the damage caused by periodontitis (6-8).

The adjunct use of growth factors in periodontal regenerative therapies has had increased interest among clinicians. Platelet derived growth factor (PDGF), Bone Morphogenetic Proteins (BMP) and Enamel Matrix Proteins are among some options utilized to speed and optimize hard or soft tissue regeneration (9-12). However, data is still conflicting in regard to clinical and radiographic outcomes when these signals are applied (13).

Platelet rich plasma (PRP) is a platelet concentrate developed from autologous blood and obtained by a gradient density cell separator. It has been widely used in medicine and dentistry with regenerative purposes due to its angiogenic and osteogenic potential and high growth factor release. However, the liquid nature of the PRP and its short-living effects imposes limitations to its application.

Injectable hydrogels have been attractive scaffolds in 3D cultures studies with attracting properties such as high permeability for oxygen, nutrients and water. In vivo, the gel state of the scaffold has been linked to several advantages such as filling the defect right after gelation therefore reducing undesirable diffusion of the gel into the surrounding tissues (14). A double cross-linked biopolymer known as gelatin hydroxyphenil propionic acid (Gtn-HPA) has been investigated to serve as a carrier / scaffold in tissue engineering. This novel hydrogel can undergo crosslinking *in situ* and degraded by host cell enzymes within 1-2 weeks (15).

In the present study, we explored the use of a novel injectable hydrogel incorporating PRP by testing the hypothesis that the novel 2% Gtn-HPA hydrogel is an efficient method for human PRP delivery and enables enhanced regenerative potential in osteoprogenitor cells and also stimulates growth factor release.

REVIEW OF LITERATURE

1. Periodontal disease, regeneration and tissue engineering

Chronic periodontitis is among the most prevalent diseases in the world and it affects more than 50% of the Americans. Among the mild, moderate, and severe forms of the disease, the prevalence of moderate periodontitis is the highest, affecting 30% of adults over 30 years of age (16). The etiology of periodontitis is biofilm accumulation around natural teeth (1, 2) in a susceptible host and as a result of this infectious disease an inflammatory process develops leading to loss of periodontal ligament, cementum and supporting bone around teeth. The greatest potential of periodontal regeneration is achieved when these defects are managed at an early stage (17). However, chronic periodontal diseases are not painful so often times the disease is only diagnosed at an advanced stage, limiting regenerative outcomes with non-surgical therapies. Besides the impact on human health, in the USA it is estimated that the annual cost for treating periodontal diseases exceed 14 billion dollars (18).

The term regeneration means the restoration of the lost part (19). In the periodontium, regeneration means the formation of new bone, cementum and periodontal tissue in a root previously exposed to bacterial contamination (20). In a series of studies, Bowers et al (6, 7) were the first authors to describe histological evidence of regeneration

following periodontal disease as they observed more new attachment apparatus formation on grafted sites vs. non-grafted sites. Since then, multiple studies have investigated the regenerative effects of the use of bone grafts, growth factors, scaffolds and combination therapies such as guided tissue regeneration (GTR) for treating furcation defects and bone craters (infrabony defects) caused by the periodontal tissues breakdown (12, 21, 22). Besides having many materials and different treatment options in the clinicians' table, many can be considered expensive, technique sensitive and with limited evidence of periodontal regeneration (17).

The goal of tissue engineering and regenerative medicine is to promote regeneration and restore a tissue's structure and function more predictably, more quickly and less invasively than other techniques. Therefore, it is important to understand the molecular and cellular mechanisms involved in the healing process in order to promote healing and regeneration, optimizing the treatment's outcomes. The 3 key elements of tissue engineering aiming periodontal regeneration include: (1) conductive scaffolds or matrix, (2) signaling molecules and (23) cells. Many bioactive proteins in bone, cells, platelets and tissues can work as signaling molecules (24). As periodontists continue to seek for regenerative modalities that are less technique sensitive, lead to faster results and are aplicable to a broader array of clinicians (25), the use of tissue engineering in respect to autologous materials has raised interest among the field.

2. **Growth Factors**

As mentioned before, many bioactive proteins can act as growth factors or morphogens, having mitogenic and chemotactic properties, allowing both cell proliferation and cell migration, respectively. Growth factors express normal gene function, act in the external membrane receptors, and are not mutagens, therefore not posing a risk for hyperplasia or neoplasia. They can also regulate biosynthesis and wound healing (24, 26).

Recombinant human PDGF-BB (rhPDGF-BB) was the first FDA (Food and Drug Agency) approved growth factor for bone and periodontal regeneration, allowing for a 1,000-fold concentration of PDGF than seen in the whole blood platelet content. Rh-PDGF-BB also presents mitogenesis and chemotaxis properties, stimulating cell division and migration (27). It is considered the current standard for biological growth factor use in periodontal and peri-implant regeneration (28). Its regenerative potential has been extensively proven in many in vitro, pre-clinical and clinical studies including Class-II furcations, interproximal bone defects, among others (12, 17, 28, 29). However, PDGF's relatively high cost and limitation due to food and drug agencies may diminish its potential clinical application worldwide.

During natural wound healing, blood plays a major role in providing cells, growth factors, cytokines and coagulation factors (30). Growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF-B) are known to be released by platelets. Finally, in 1998, Marx et al introduced the use of concentrated forms of

platelets termed Platelet Rich Plasma (PRP) to be used in dental surgical procedures expanding the regenerative possibilities using autologous blood (26).

3. **Platelet Rich Plasma**

Platelets are known for their role in coagulation and homeostasis, leading to thrombin generation and fibrin formation but they can also promote tissue repair and influence angiogenesis and inflammation. Therefore, the use of platelet aggregates could positively influence clinical situations requiring faster healing and tissue regeneration (31). In addition to its clinical application in the context of various orthopedic diseases, PRP has been in focus in many fields of tissue engineering (32).

Platelet derivatives can be classified based on two main parameters: fibrin and leukocyte contents, as shown in figure 1. Our focus in this review is to discuss the findings related to L-PRP, or simply PRP.

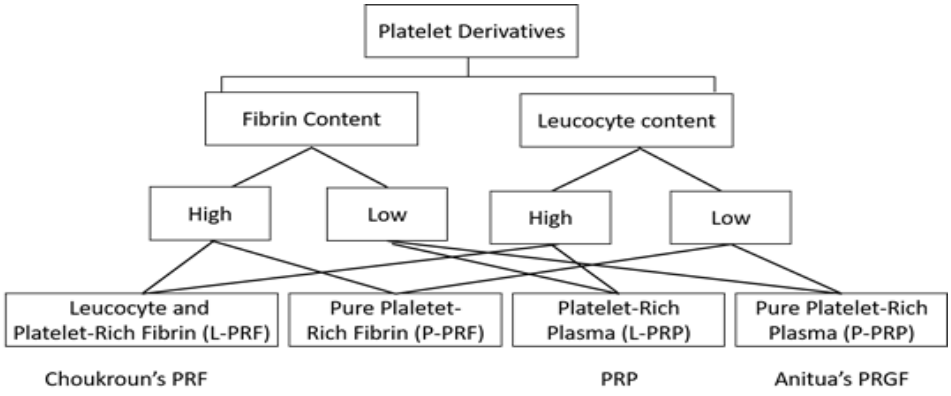

Fig. 1. Platelet derivatives classification (33).

Platelet-rich plasma (PRP) refers to an enriched concentration of autologous platelets suspended in a small amount of plasma after centrifugation (30), whereas the final platelet concentration is several folds above the physiologic levels (34). PRP was first described by Marx, in 1998, and it was the first autologous growth factor source introduced in the dental field. Its use has widespread ever since due to its biological efficacy, safety and effectiveness (24).

With PRP, the concentration of platelet often reaches 3-10 times compared to that of whole blood (30, 35). Most PRP protocols call for the use of anticoagulant when collecting blood from patients. To obtain the PRP, the collected blood undergoes two-step centrifugation at varying speeds. After the first spin (often shorter in duration with lower centrifugal force), blood separates into 3 days: platelet poor plasma (PPP), buffy coat (containing leukocyte and platelet) and red blood cells. The top two layers containing plasma, leukocytes and platelets are collected into another tube without anticoagulant and undergo a second spin with longer duration (Figure 2). For the maximum strength in release of growth factors, the resultant PRP should be activated prior to its application, to reverse the anticoagulant effects and initiate the cascade of coagulation leading to clotting. Often times this activation is obtained with bovine thrombin and calcium chloride (CaCl) (36, 37),(24, 33).

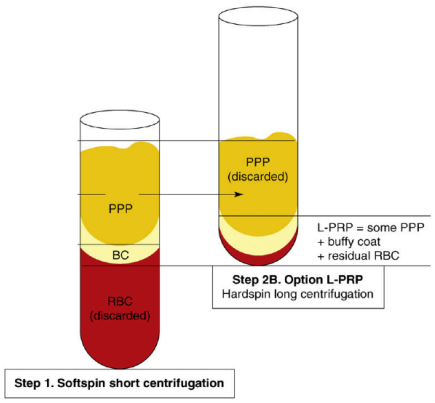

Fig. 2. Two-step centrifugation protocol to obtain PRP (Anitua et al, 1999).

The platelets' alpha-granules content is vast (Figure 3). Studies have shown that the platelets in PRP degranulates and the growth factor release starts in the first 10 minutes, having 90% of total secretion within an hour (38). This burst of growth factors release intensifies the signal to the local mesenchymal and epithelial cells to migrate, divide and increase collagen and matrix synthesis, resulting in faster healing and higher bone formation. After wound closure, the platelets continue to synthesize and secrete more growth factors for up to 10 days (24, 30)

Growth factors	Function	Mechanisms	References
Transforming growth factor-β (TGF-β)	Stimulates endothelial chemotaxis and angiogenesis Regulates mitogenic effects of other growth factors (nerve growth factor, brain derived neurotrophic factor, etc) Induces undifferentiated mesenchymal cell proliferation Stimulates neurite outgrowth and regulates differentiation of SCs Inhibits macrophage and lymphocyte proliferation.	Secrets into the local nerves by damaged neurons, invading macrophages and degenerated schwann cells (SCs) during Wallerian degeneration Activates SCs and recruit macrophages to the injury site of distal nerve stumps	Unsicker and Strelau[41] Gordon *et al.*[42] Sulaiman and Dreesen[43]
Platelet-derived growth factor (PDGF)	Stimulates trophic activity on neurons Induces SCs proliferation, differentiation and myelin formation Mitogenetic for mesenchymal cells and osteoblasts. Involved in the wound healing of hard and soft tissues and for central nervous system development Stimulates hyaluronan and glycosaminoglycans (components of the extracellular matrix)	Acts on the factor tyrosine kinases (RTKs) and the downstream PI3K and mitogen-activated protein (MAP) kinase signaling pathways Stimulates chemotaxis of neutrophils and macrophages to the injured sites and participates in tissue re-epithelialization and/or angiogenesis	Graham *et al.*[44] Yamazaki *et al.*[45] Burnouf *et al.*[46] Hellman *et al.*[47]
Vascular endothelial growth factor (VEGF)	Enhances angiogenesis and vessel permeability Regulates cell proliferation and mediates antiapoptotic effect Stimulates mitogenesis for endothelial cells Promotes blood vessel growth, neurogenesis and neuroprotection	Induces injury-related angiogenesis by connecting to endothelial transmembrane receptors identified as fms-like tyrosine kinase (FLT-1), fetal liver tyrosine kinase receptor (FLK-1) and neurophilin-1	Mackenzie and Ruhrberg[48] Hermann and Zechariah[49]
Insulin-like growth factor-1 (IGF-1)	Initiates the formation of bud growth Supports the forward extension of the nerve fibers Suppresses apoptosis in motor, sensory and sympathetic neurons Stimulates protein synthesis	Via the PI3K pathway, induces SCs to synthesize of two fatty acids that initiates the process of myelination Secreted in the vicinity of the injured nerve sites and its receptors are mainly expressed on axon, nerve terminals, SCs and motor neuron cell bodies, promoting early recovery of sensation	Liang *et al.*[50] Nagata *et al.*[51]
Basic fibroblast growth factor (bFGF)	Contributes to embryonic development, angiogenesis and wound healing Facilitates neuroprotection and SCs regeneration	After peripheral nerve injury, bFGF and its high-affinity tyrosine kinase receptor FGFR-3 are upregulated in sensory neurons and at the lesion site of the nerve, inducing angiogenesis and accelerates wound closure	Grothe and Nikkhah[52]

Fig. 3. Platelet alpha-granules content and their functional categories (39).

There is a vast literature focusing on PRP use in different periodontal procedures involving both hard and soft tissue augmentation. As discussed before, the purpose of using PRP in periodontal procedures is to attempt enhanced and faster wound healing and the maturation of bone. It is considered a promising biomaterial due to its osteoinductive properties, easiness to handle, availability in large scale and reduced risk of disease transmission or immunogenic reactions (34). Among many growth factors present in PRP, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular

endothelial growth factor (VEGF), and transforming growth factor (TGF-B) exert great influence on bone regeneration. PRP also enhances wound healing and facilitates cell proliferation, chemotaxis and differentiation (34, 40).

Soffer et al, 2003 demonstrated the advantage of using PRP for bone augmentation and it was seen that by using PRP to introduce PDGF, TGF-B, and IGF-1 the natural blood clot was enriched, and a stimulation of bone regeneration occurred (41). Albanese et al in 2013, discussed the advantages of PRP in wound healing such as: the adhesive ability of PRP facilitated easier manipulation of membranes, better flap adaptation and hemostasis, and a better closure. They concluded that the use of PRP can extend beyond periodontal regeneration to mandibular reconstruction surgery, surgical repair of various alveolar cleft deformities, and also possible management of bisphosphonate-related osteonecrosis of the jaw (BRONJ) or avascular necrosis (42). Also, many data suggest that PRP combined to bone grafts promote greater bone regeneration, higher percentage of vital bone and better wound healing (43), but literature is still conflicting (40, 44-48). These results can be related to mislead selection of biomaterials, inadequate animal model, improper clinical application of the growth factor, fast degradation rate of PRP and sub-optimal PRP preparation. Another drawback worth to mention is that PRP is liquid by nature requiring the combination with other biomaterials such as bone grafts (30).

As discussed above, clinical application of PRP often show conflicting results, which most likely can be attributed to the lack of standardized PRP preparation methods,

particularly in regard to the platelet count and individual patient response. Therefore, the use of PRP has been limited due to variable outcomes and multiple PRP preparation techniques. However, the use of PRP may allow us to progress to new active treatments, providing more efficient and predictable outcomes to our patients (24). Ultimately, to obtain optimal results, the use of PRP has to be individualized for the human application and more studies need to be conducted to standardize PRP protocols and establish more efficient delivery systems for PRP.

4. **Gtn-HPA hydrogels**

Many of the commercially available scaffolds used aiming growth factors release lack the ability to recruit cell adhesion molecules, resorb too fast, and cannot control the soft tissue healing. It may be said that they may be good carriers, but not necessarily good matrices (24).

The majority of the carbohydrate, protein or synthetic-polymer based hydrogels are not able to be injected as liquids and then sub-sequentially become solid in vivo (15), limiting therefore its application as a scaffold and carrier. Injectable hydrogels, often referred as gelatin or simply gel, have been proposed as scaffolds for tissue regeneration. Some advantages include its high permeability to nutrients and oxygen favoring cell growth and differentiation (14, 49). It is also able to modulate the environment and fill the defect in a non-surgical approach.

Recently, a covalent cross-linked injectable biopolymer known as gelatin-hydroxyphenyl propionic acid (Gtn-HPA) was first developed and investigated for central nervous system regeneration after brain injuries. It demonstrated high gelation rate, cytocompatibility and neural stem cells (NSCs) presented increased resistance to oxidative stress, greater viability, higher proliferation and migration to the hydrogel compared to non-crosslinked hydrogels (49, 50). Gtn-HPA has also shown compatibility with retinal progenitor cells (RPCs) as seen by the high rates of cells survival and proliferation and minimal apoptosis.

The biopolymer Gtn-HPA is a novel resorbable hydrogel that can cross-link *in situ* (Figure 4). It utilizes a time-sensitive double cross-linking reaction catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP) whereas HRP controls gelation time and H_2O_2 controls stiffness degree. After gelation and transplantation, it can be degraded in 1-2 weeks by host and donor cell enzymes (15).

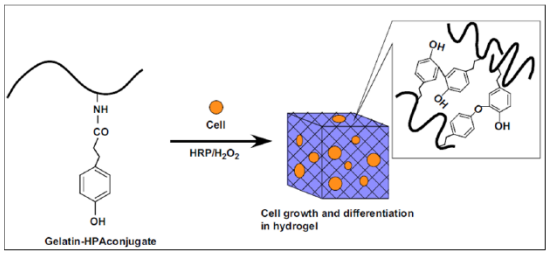

Fig. 4. Chemical structure and cross-linking process of gelatin hydroxyphenyl propionic acid (Gtn-HPA).

The advantages seen in Gtn-HPA such as the cross-linking in situ, the control of the gelation rate and stiffness plus the quick degradability makes this biomaterial a promising scaffold to be tested in conjunction to PRP to evaluate the response of osteoprogenitor cells as well as to improve the delivery system allowing different therapeutical approaches. Our long-term goal is to identify the best condition to apply the Gtn-HPA associated to a standardized clinical PRP protocol in order to utilize this scaffold incorporated into the platelet concentrate as an injectable biomaterial aiming periodontal regeneration in vivo.

SIGNIFICANCE

It is well established that regenerative procedures are capable of improving tooth prognosis. Therefore, the clinician needs to decide what is best treatment option for his patient amongst the regenerative arsenal. The use of biologics such as recombinant human PDGF (rh-PDGF) and enamel matrix derivative have shown comparable outcomes to guided tissue regeneration and bone grafts. Laser has also shown histologic evidence of regeneration. However, all of these procedures and / or biomaterials involve surgical access which always imposes risks to the patient and extends the duration of the treatment.

Thinking about the reliability, safety and easy access to our patients' blood, our future goal is to develop a reliable, safe, easy, reproducible and cost-effective protocol using injectable Gtn-HPA incorporating PRP for periodontal regeneration in a non-surgical approach, in a similar manner as local antibiotics are applied into periodontal pockets. Even more, the use of an injectable hydrogel that can crosslink *in situ* is desirable in order to maintain the gel into the defect for a longer period of time than seen with the liquid nature of PRP alone. Thus, shallow / small periodontal defects could be treated in the initial phases of the disease reducing therefore the need for surgical therapy.

INNOVATION

A critical goal of regenerative medicine is to understand and to subsequently imitate the mechanisms of tissue development and homeostasis. Although it is well known that PRP is an osteoinductive material, little is known about the effects of PRP at the gene level. Therefore, in this study we proposed to study the gene expression of osteoprogenitor cells for genes encoding osteoblast differentiation markers (OCN, Col1A1), and growth factor receptors (FGFr, EGFr and PDGFr). In this sense, we can see the bone formation potential of this PRP protocol and also try to relate the growth factor release data to the gene level.

Another point to mention is that PRP's liquid nature many times limits its use by the clinicians. Also, a precise protocol for PRP remains poorly defined with multiple methodologies observed between studies. Therefore, it is empirical to:

1) include a scaffold in the PRP protocol (injectable, preferably) to release the PRP in a controlled and secure manner;
2) standardize a protocol for reproducibility, including patient platelets analysis and centrifugation time and setup.

Therefore, the use of our PRP clinical protocol* added to the novel Gtn-HPA herein used as an injectable carrier seems to be a desirable approach worthy to be investigated.

* Clinical protocol for PRP preparation currently used in the Periodontology Clinic at Harvard School of Dental Medicine, according to Intini et al, 2007.

HYPOTHESIS AND SPECIFIC AIMS

We hypothesized that the novel Gtn-HPA hydrogel is an efficient method for human PRP delivery, enabling enhanced regenerative potential in osteoprogenitor cells and also stimulating growth factor release. We addressed this hypothesis in the following 2 specific aims:

- **Specific Aim 1: Detect and quantify the protein levels of growth factors released by the Gtn-HPA incorporated with PRP.** PDGF, TGF-B, FGF, EGF and IL1-B will be analyzed by Enzyme-Linked Immunosorbent Assay (ELISA);

- **Specific Aim 2: Evaluate and quantify the gene expression of osteoprogenitor cells in the Gtn-HPA incorporated with PRP.** OCN, Col1A1, PDGFr, FGFr and EGFr genes will be analyzed by real time quantitative polymerase chain reaction (RT-qPCR).

MATERIAL AND METHODS

- **Platelet rich plasma (PRP) preparation**

For this study, blood samples were harvested from 6 voluntary donors that approved and signed an informed consent allowing the use of the discarded PRP prepared for surgical periodontal procedures in the Periodontology Clinic at Harvard School of Dental Medicine (HSDM). The blood used was obtained from donors that would have periodontal surgical procedures using PRP and the remaining discarded amount was collected to be used in a non-identifiable manner; therefore, the research project was approved by IRB with an expedite basis. All samples were obtained from donors between the ages of 28 and 50.

The PRP was prepared following the protocol used in the Periodontology Clinic at Harvard School of Dental Medicine (Intini et al. 2007), as described step-by-step and illustrated below (Figure 5):

1. Connect the 20G blood collection needle to the vacutainer holder;
2. Use the tourniquet to constrain the blood flow on the R or L arm of the patient;
3. Locate the cephalic or basilic vein;
4. Using 2 vacutainer tubes with the yellow cap (containing anticoagulant [ACD] solution A), withdrew a total of 18cc of blood (~9cc of blood for each tube since 1cc is anticoagulant, making a total of 10cc per tube);
5. Place both tubes in the centrifuge opposing to each other and centrifuge for 2 minutes and 15 seconds (please note that this protocol works only with a centrifuge

able to generate a force of 1000g; the centrifuge used has a radius of 7.5cm and spins at 3600rpm which generates 1000g);

6. Remove tubes from centrifuge and remove the yellow caps from each tube;
7. Attach the aspirating needle (16g x 3.5 inches) to a 10cc syringe and aspirate the supernatant composed of plasma, platelets and WBC from both tubes;
8. Transfer the supernadant from both tubes to one new single vacutainer tube with a red cap (without anticoagulant). In this tube, 6-8cc of supernadant will be seen (plasma, platelets and WBC);
9. Prepare another red cap tube for balancing the next centrifugation by doing the following: take out the cap from the tube and add water to it to a level comparable to the level reached by the supernatant contained in the other tube in the centrifuge and should be discarded immediately afterward);
10. Place both the tube containing the supernadant and the balancing tube in the centrifuge (facing each other) and spin for 5 minutes;
11. Remove both tubes from the centrifuge, discard the once containing water;
12. Take the tube containing the supernadant: a small pellet of platelets and WBCs will be seen at the bottom of the tube;
13. Using the same aspirating needled attached to a 10cc syringe, aspirate the yellow liquid (serum) from the tube. During this aspirating procedure, avoid disturbing the pellet. Aspirate the amount necessary to leave in the tube approximately 1cc or serum together with the pellet;
14. Discard the serum that has been aspirated;
15. Using the same aspirating needle, re-suspend the pellet into the 1ml of serum remaining in the tube, gently aspirating up and down the serum for approximately 5-8 times;
16. Now the PRP is ready to be used alone or mixed with other biomaterials for clinical purposes. **(Note that this step was not performed in this study)*.

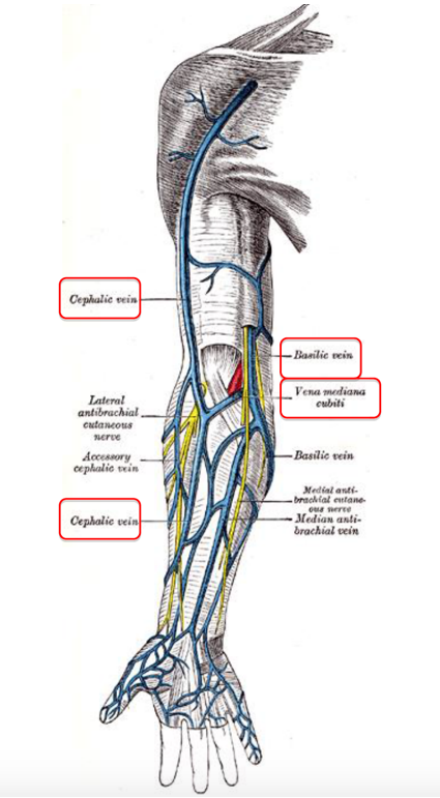

Fig.3. Anatomical sites used for blood drawing

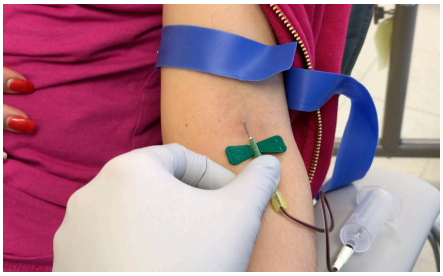

Fig.4. Blood being drawn for PRP preparation.

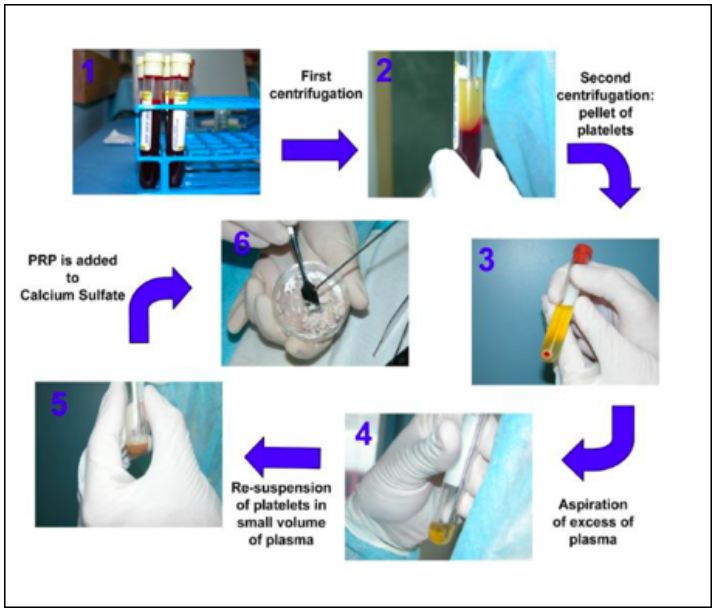

Fig.5. Platelet Rich Plasma (PRP) preparation (Intini et al, 2007)

- **Platelet counting**

Prior to addressing the specific aims, hematology analysis was performed in order to quantify the platelet counts for whole blood and PRP amongst the donors (n = 6). For this purpose, whole blood samples and PRP vials were sent out for platelet counting quantified by a hemocytometer at the Hematology Department at the Veterans Affairs Boston Healthcare System, Jamaica Plain/MA. The platelet count was performed between PRP preparation and PRP activation.

As a sub-aim of this study, we wanted to evaluate if the initial blood count interferes in the final platelet concentration seen in the PRP as well as in the growth factor release and gene expression profile outcomes.

- **PRP activation**

After PRP preparation, the PRP was double activated with thrombine and calcium chloride, as described by Marx et al, 1998. Thrombine was added in a concentration of 142.8U/ml and calcium chloride at 14.3mg/ml. The PRP lysate was obtained after 1-hour incubation at 37ºC in a 5% CO_2 chamber.

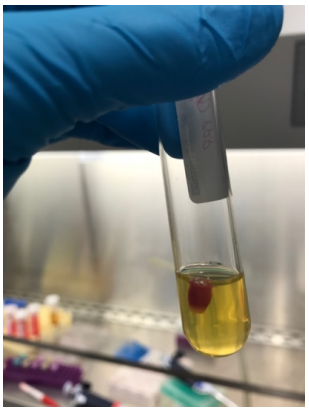

Fig.6. PRP lysate after 1-hour incubation at 37ºC in a 5% CO_2 chamber.

- **Osteoprogenitor cells**

In this study, osteoprogenitor cells were used for the cell viability and RT-qPCR experiments. Immortalized cells from mouse bone marrow/stroma identified as W-20-17 [W-20 clone 17] (ATCC CLR 2623^{TM}) were used and cultured in high glucose (4500 mg/L glucose) Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% Fetal Bovine Serum (FBS) and 1% antibiotic/antifungal.

- **Hydrogel preparation (Gtn-HPA)**

Lyophilized Gtn-HPA was obtained from our collaborator at the Institute of Bioengineering and Nanotechnology in Singapore where it was synthesized as previously described by Hu et al, 2009 (51).

While the PRP was being activated, the hydrogel preparation was carried out. The Gtn-HPA was prepared in a 2% concentration and double cross-linked with HRP (0.1 U/ml) and H_2O_2 (1.2 mM) for 2 minutes. From previous studies in our lab, we know that the 2% Gtn-HPA concentration is permissive for cell proliferation and migration, and no adverse effects of the gel is seen on the viability of the cells.

In order to confirm the cell viability of the 2% Gtn-HPA hydrogel, we used W20 clone 17 cells (ATCC CRL-2623) in 3 distinctive groups: control (no PRP), 10% PRP and 20% PRP (n=1). The control cells were seeded in a monolayer fashion and for the 10% and 20% PRP groups the cells were seeded on top of the gel with the density of 20,000 cells/well, with DMEM-HG + 2% FBS. Casted 300µl 2% Gtn-HPA in 50% PBS + 50% DMEM-HG into each well of 12 well-plate, crosslinked with 0.1U/ml HRP + 1.2nM H_2O_2. Cells were then cultured overnight, stained with Calcin AM (live, green, 250 ng/ml) and EthD-1 (dead, red, 1µM) for 40 mi, then rinsed in PBS for 15 min. Fluorescence imaging was followed and viability was calculated: viability= live cell number / (live + dead) cell number.

- **Protein quantification (ELISA)**

The enzyme-linked immunosorbent assay (ELISA) was carried out to quantify the total amount of growth factors released from PRP incorporated into a 2% Gtn-HPA at 15 min (n = 5). The PRP lysates were harvested from standard tissue culture media, then collected and frozen for future processing. Standing ELISA was utilized to quantify the levels of Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Endothelial Growth Factor (EGF), Tumor Necrosis Factor alpha (TNF-α), and Interleukin 1 beta (IL-1β) according to the kit manufacturer's protocols (PDGF, FGF, EGF and IL-1β: PrepoTech, NJ, USA; TNF-α: R & D Systems, MN, USA). Absorbance was measured at 450 and 570 nm on an EL x 808 Absorbance Reader (BIO-TEK, VT, USA) and samples were quantified in triplicate.

- **Real-Time Polymerase Chain Reaction (RT-qPCR)**

Gene expression analysis was performed in 6 experimental groups (n = 3), in triplicate:

1. 2D: cells in monolayer (2% FBS medium), no PRP
2. 2% Gtn-HPA
3. 2% Gtn-HPA + 10% FBS
4. 2% Gtn-HPA + 20% FBS
5. 2% Gtn-HPA + 10% PRP
6. 2% Gtn-HPA + 20% PRP

For groups 2 to 6, the osteoprogenitor cells (20 x 10^3 cells / ml) were seeded on top of the gel. Then, cells in all groups were allowed to proliferate for 2 days.

Total RNA was harvested 48h after stimulating the osteoprogenitor cells to investigate mRNA levels of osteoblast differentiation markers: Osteocalcin (OCN) and Collagen type 1 alpha 1 (Col1A1), and the growth factor receptors: Fibroblast Growth Factor receptor (FGFr), Endothelial Growth Factor receptor (EGFr), and Platelet Derived Growth Factor (PDGFr). First, conventional RNA isolation with Trizol® (Thermo Scientific, Wilmington, DE) was performed, followed by cDNA synthesis and RT- qPCR (TaqMan Fast Advanced Master Mix, Thermo Scientific, on an Applied BioSystems 7500 RT-qPCR machine) was then performed according to fabricator's instructions. A Nanodrop 2000c (Thermo Scientific, Wilmington, DE) was utilized to calculate total RNA levels. The ΔΔCt method was utilized to calculate gene expression levels normalized to the expression of β-actin (housekeeping gene).

Table 1. List of gene and codes for RT- qPCR

Gene	Gene code
OCN	Mm00649782_gH
Col1A1	Mm00801666_g1
FGFr	Mm03053754_s1
EGFr	Mm01187863_g1
PDGFr	Mm01262485_m1
β-actin	Mm02619580_g1

RESULTS

1. Platelet counting

We investigated the platelet counting for each donor to evaluate the initial whole blood platelet count and the increase in folds for in the PRP samples. This was a sub-aim of this study, to see whether we could link the initial blood counts and the final platelet concentration seen in the PRP to the growth factor release and gene expression profile outcomes.

It is known that average of platelets for a healthy adult Caucasian is between 150,000 – 450,00/ml. As we can see in Table 2, donor #7 already started with a low number of platelets, even lower than the normal range. The fold in platelet count varied significantly between donors, ranging from 3.6 to 8.7, as seen below in Figure 7. For donor #1 we were not able to collect whole blood sample from donor, therefore the platelet count for whole blood or PRP/WB fold data is not available.

Table 2. Table with total platelet counts for whole blood, PRP, and PRP/WB fold within donors.

Donor	Whole blood (WB)	PRP	PRP/WB fold
#1	-	720 x 10^3	-
#3	190 x 10^3	1,660 x 10^3	8.7
#4	147 x 10^3	1,140 x 10^3	7.7
#5	200 x 10^3	1,140 x 10^3	5.7
#6	155 x 10^3	940 x 10^3	6
#7	132 x 10^3	480 x 10^3	3.6

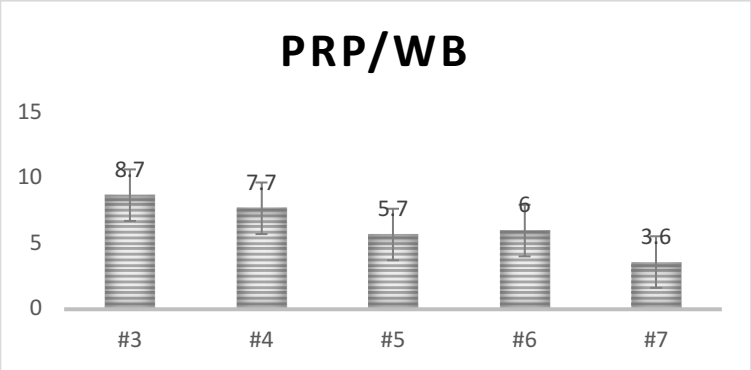

Fig. 7. Increase of platelet counts in PRP over WB. Note the significant variability within donors.

- **Cell viability**

The cell viability test was conducted as a side evaluation to confirm previous results obtained in the lab with different cell lines which revealed that the 2% Gtn-HPA hydrogel is a viable carrier, not affecting the viability of the cells.

As we can see below in Figure 8, the 2% Gtn-HPA with or without PRP did not affect the viability of the cells, whereas the green markers are viable cells. Note that in condition C, the cells seem to be inside the gel and we could speculate that the cells are migrating towards the hydrogel more expressively than in condition B.

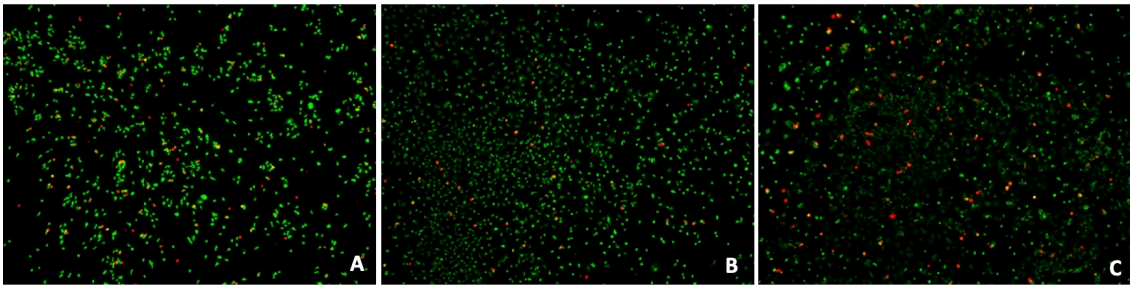

Fig.8. Viability of the 2% Gtn-HPA in 3 conditions: A) 2D culture (no PRP), 2) 10% PRP, 3) 20% PRP; n=1.

2. Protein quantification

Our proposed specific aim 1 was to detect and quantify the protein levels of growth factors released by a 2% Gtn-HPA incorporated with PRP. PDGF, TGF-B, FGF, EGF and IL1-B levels were analyzed in the PRP lysate samples by Enzyme-Linked Immunosorbent Assay (ELISA), with n = 5, and results are presented below (Figures 9-13).

We can observe that the growth factors TGF-B and PDGF were detected and released in significantly higher levels than the other growth factors analyzed here. TGF-B and EGF seems to have better consistency in detection levels compared to the other proteins. FGF and IL1-B detection levels varied the most within the donors.

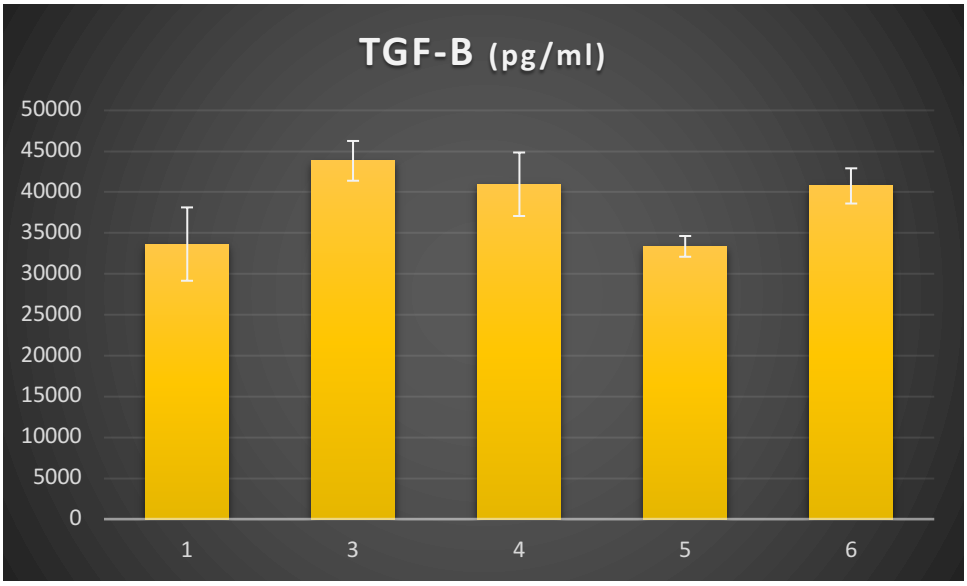

Fig. 9. Protein quantification of TGF-B, n = 5.

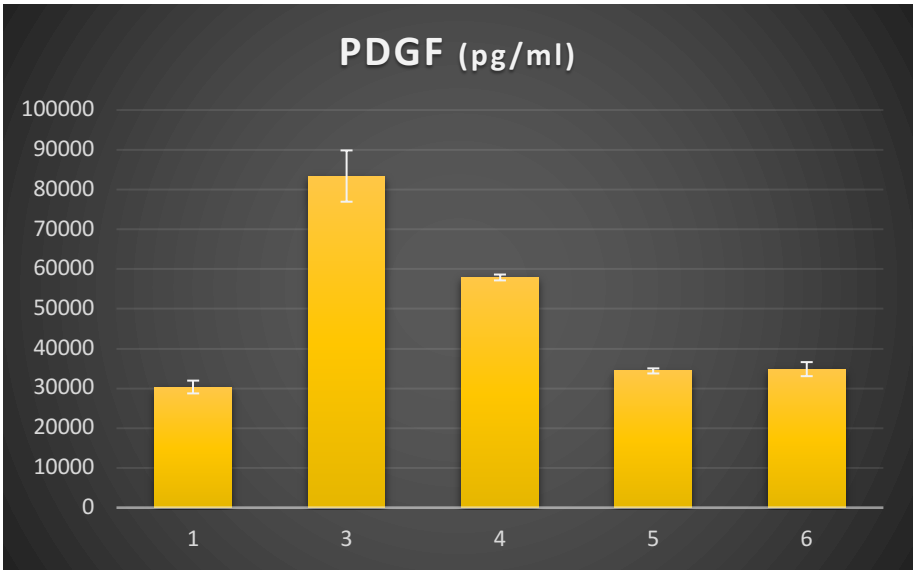

Fig.10. Protein quantification of PDGF, n = 5.

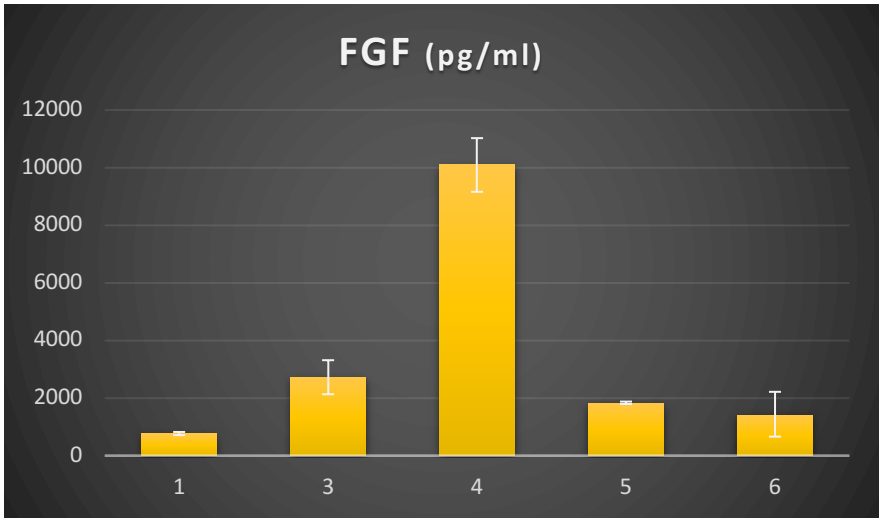

Fig.11. Protein quantification of FGF, n = 5.

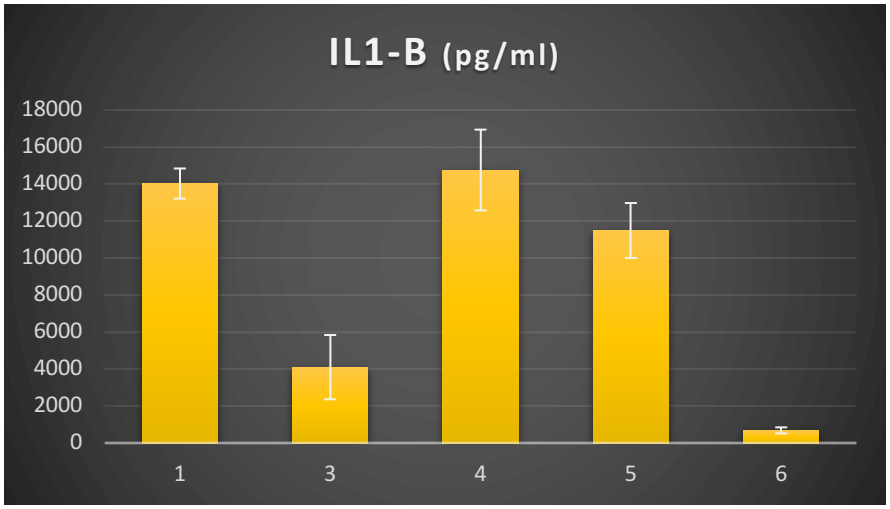

Fig. 12. Protein quantification of IL1-B, n = 5.

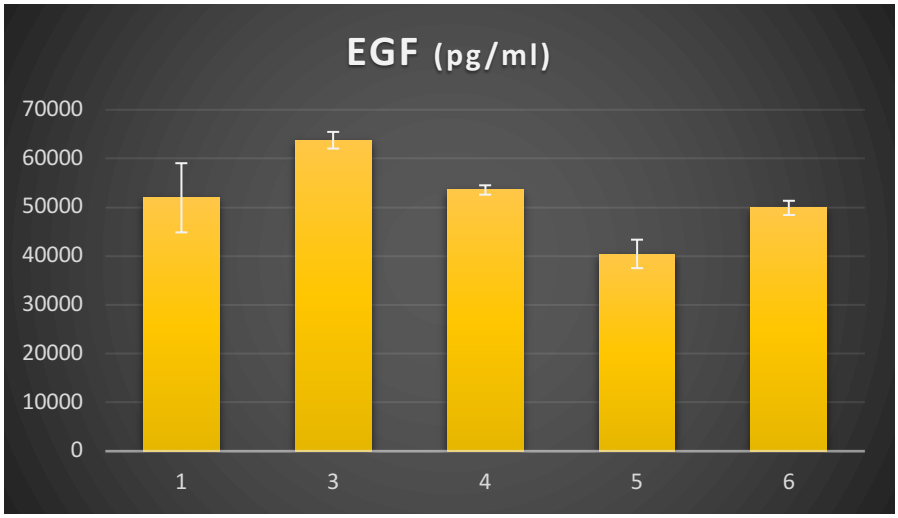

Fig. 13. Protein quantification of EGF, n = 5.

3. Gene expression

Our specific aim 2 was to evaluate and quantify the gene expression of osteoprogenitor cells in a Gtn-HPA incorporated with PRP. OCN, Col1A1, PDGFr, FGFr and EGFr genes were analyzed by real time quantitative polymerase chain reaction (RT-qPCR) using β-actin as internal control.

The expression levels of the genes related to osteogenesis were analyzed in the samples from the 6 groups described previously (2D, 2% Gtn-HPA, 2% Gtn-HPA + 10% FBS, 2% Gtn-HPA + 20% FBS, 2% Gtn-HPA + 10% PRP, 2% Gtn-HPA + 20% PRP).

Due to the great variability seen in the results between donors, the results are here presented donor-by-donor.

a) Donor #1 (PRP concentration = 720 x 10^3 platelets/ml)

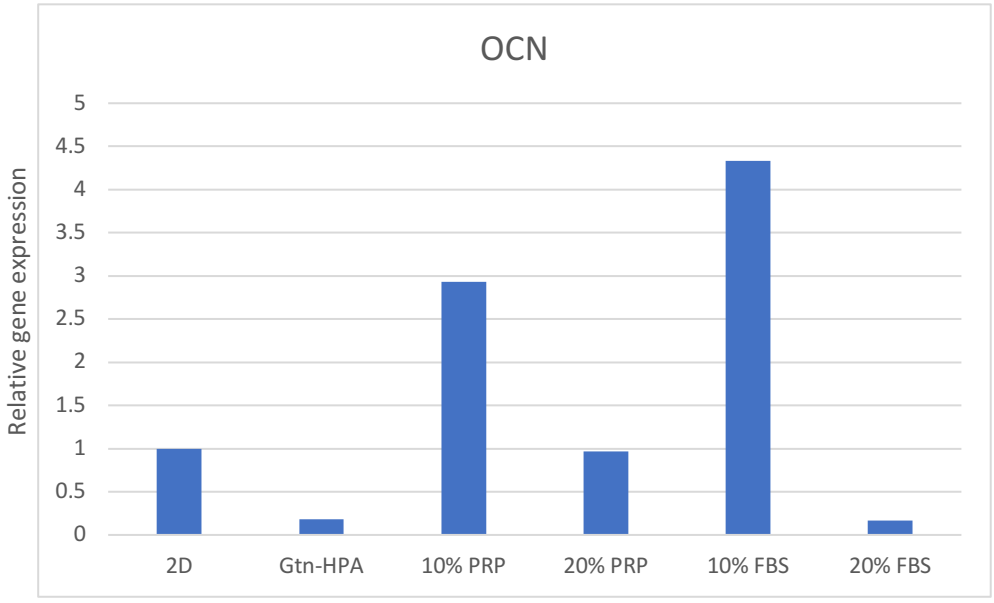

Fig. 14. Gene expression level of OCN, donor #1.

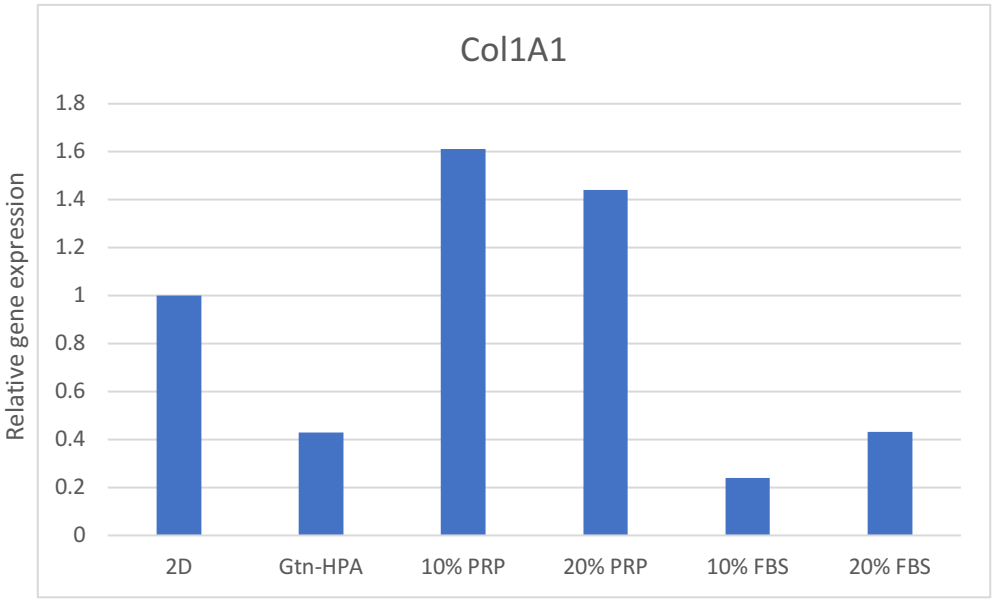

Fig. 15. Gene expression level of Col1A1, donor #1.

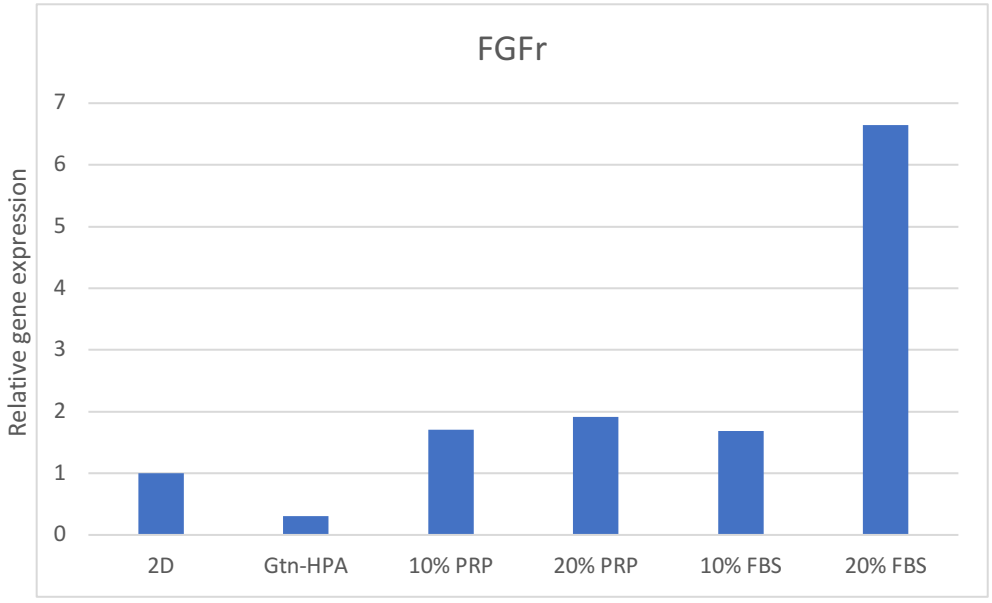

Fig. 16. Gene expression level of FGFr, donor #1.

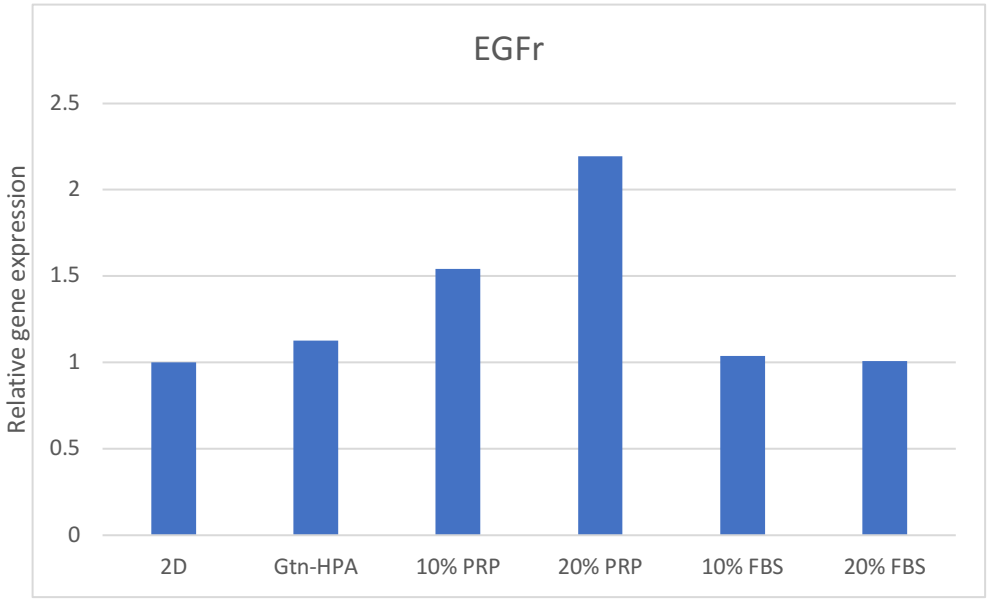

Fig. 17. Gene expression level of EGFr, donor #1.

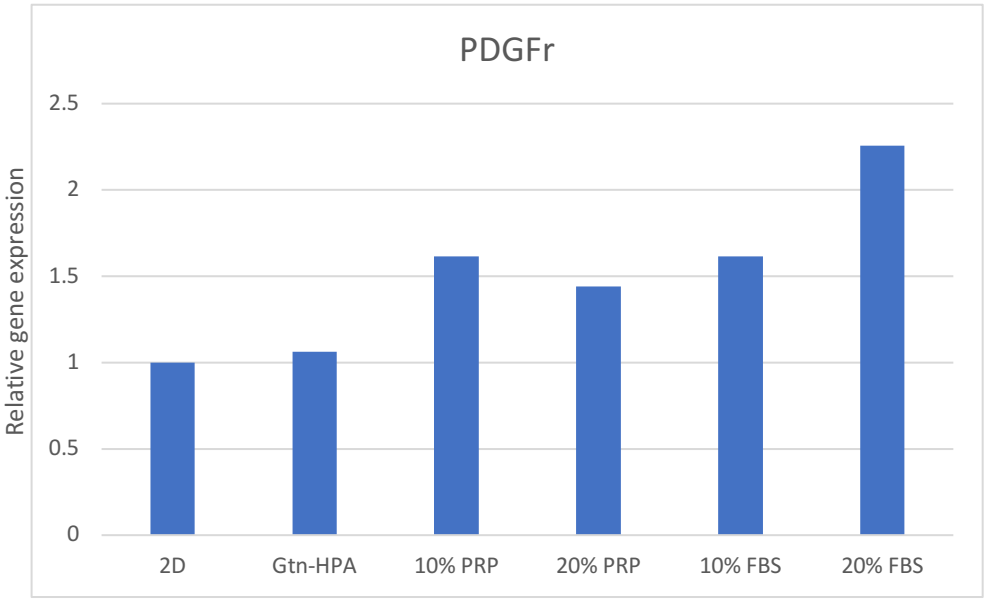

Fig. 18. Gene expression level of PDGFr, donor #1.

b) Donor #5 (PRP concentration = 1,140 x 10^3 platelets/ml)

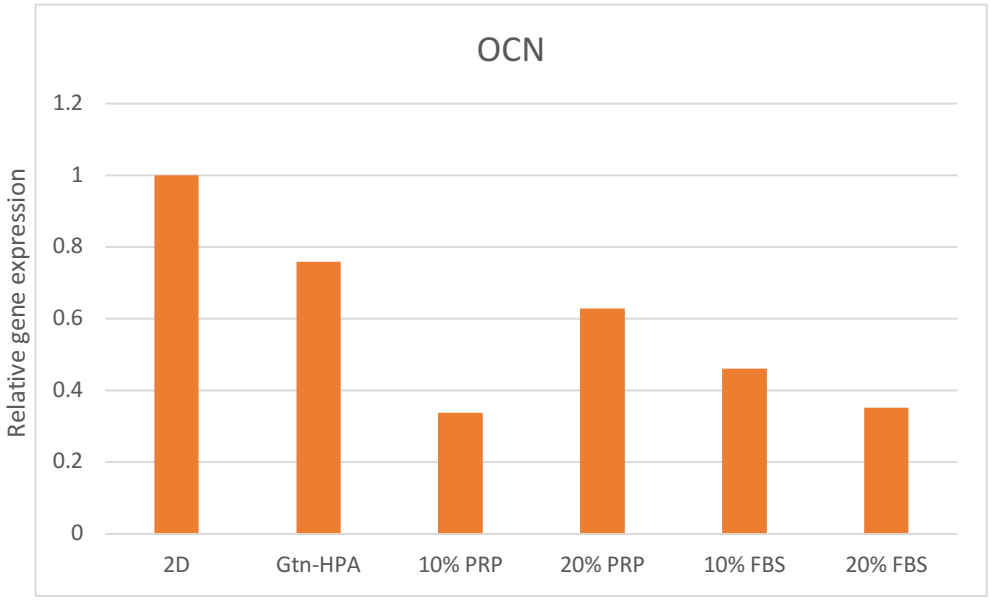

Fig. 19. Gene expression level of OCN, donor #2.

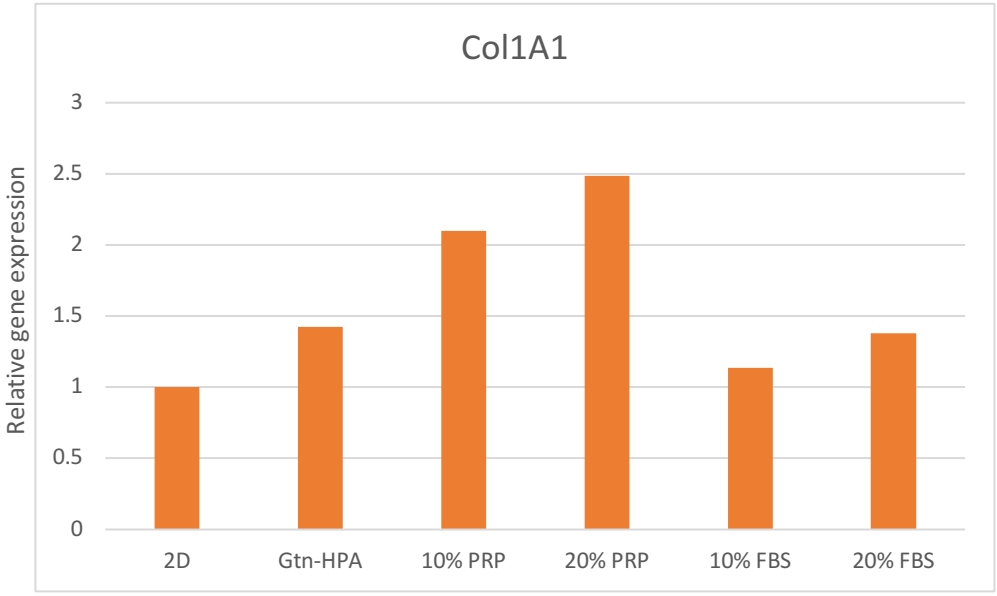

Fig. 20. Gene expression level of Col1A1, donor #2.

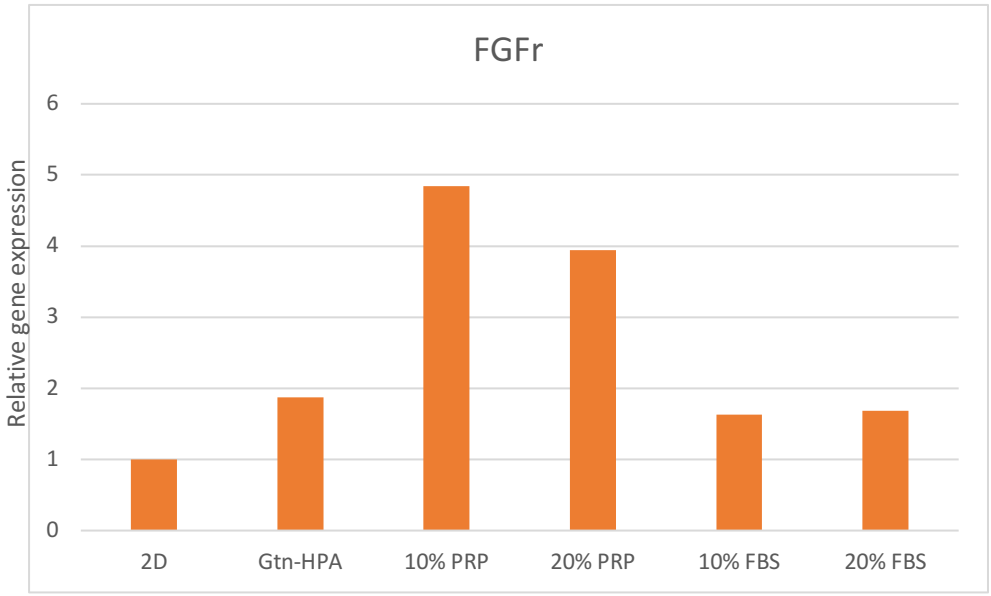

Fig. 21. Gene expression level of FGFr, donor #2.

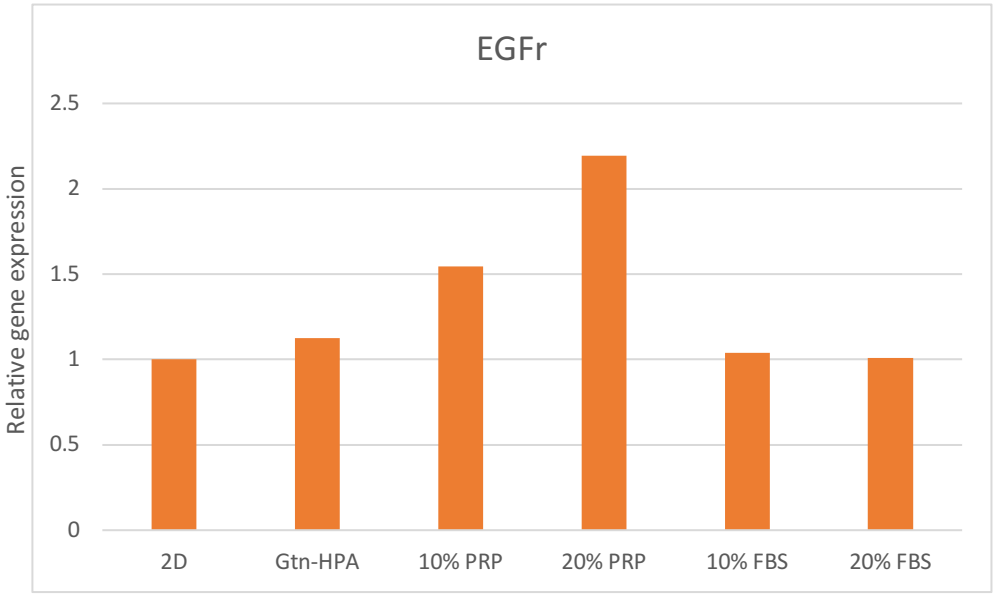

Fig. 22. Gene expression level of EGFr, donor #2.

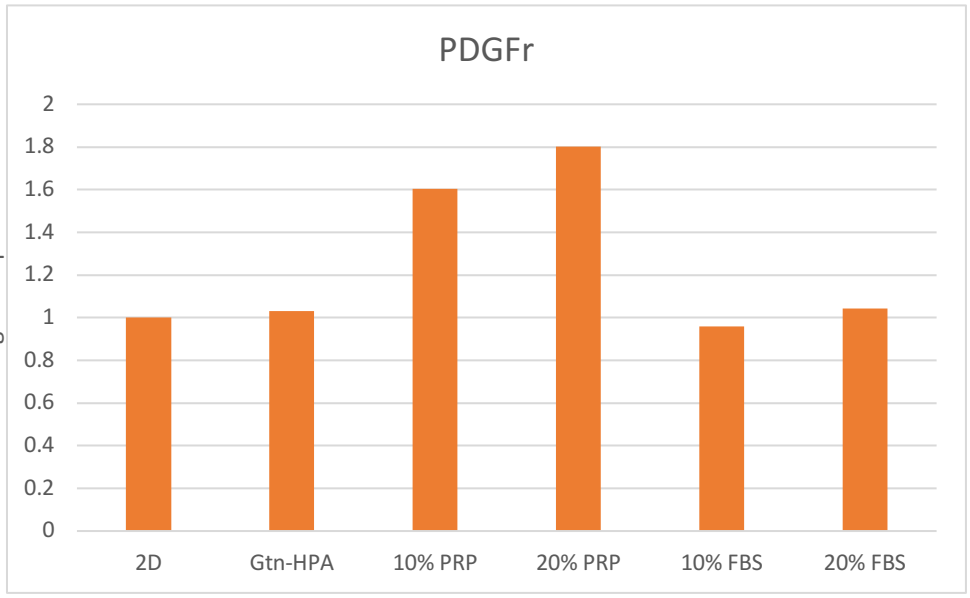

Fig. 23. Gene expression level of PDGFr, donor #2.

c) Donor #7 (PRP concentration = 480 x 10^3 platelets/ml)

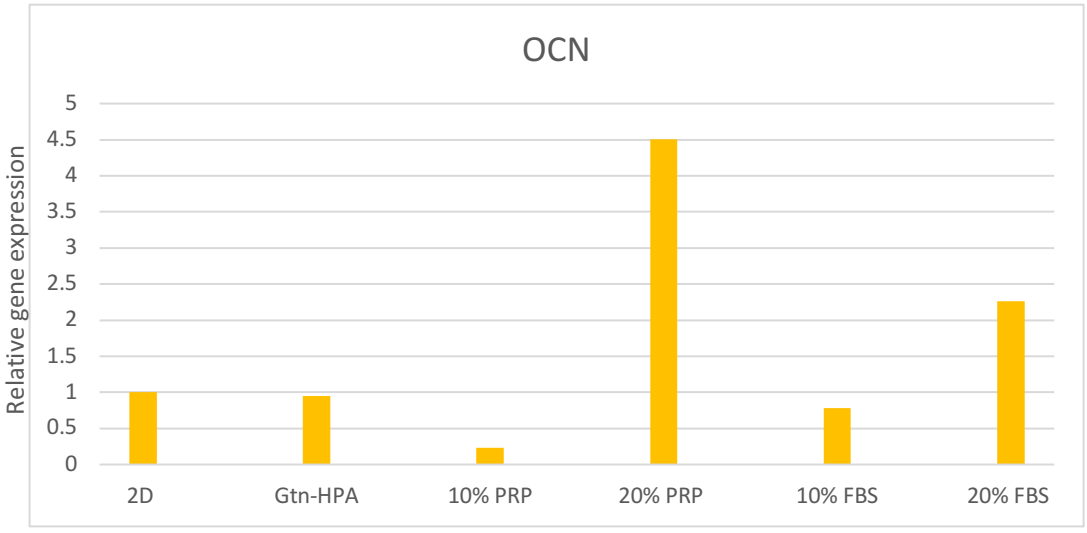

Fig. 24. Gene expression level of OCN, donor #3.

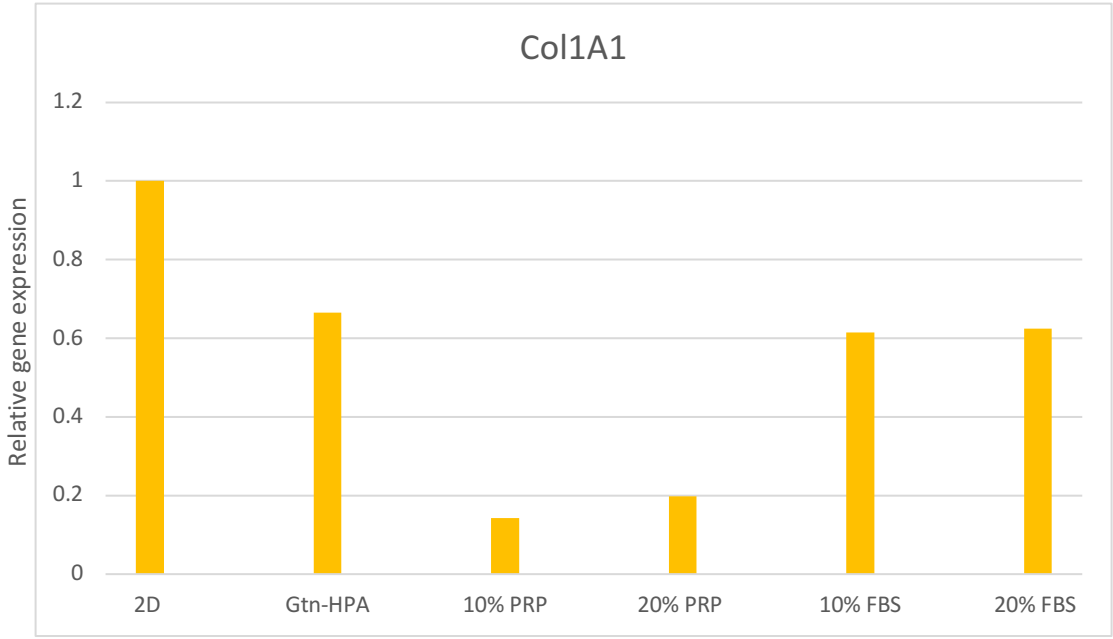

Fig. 25. Gene expression level of Col1A1, donor #3.

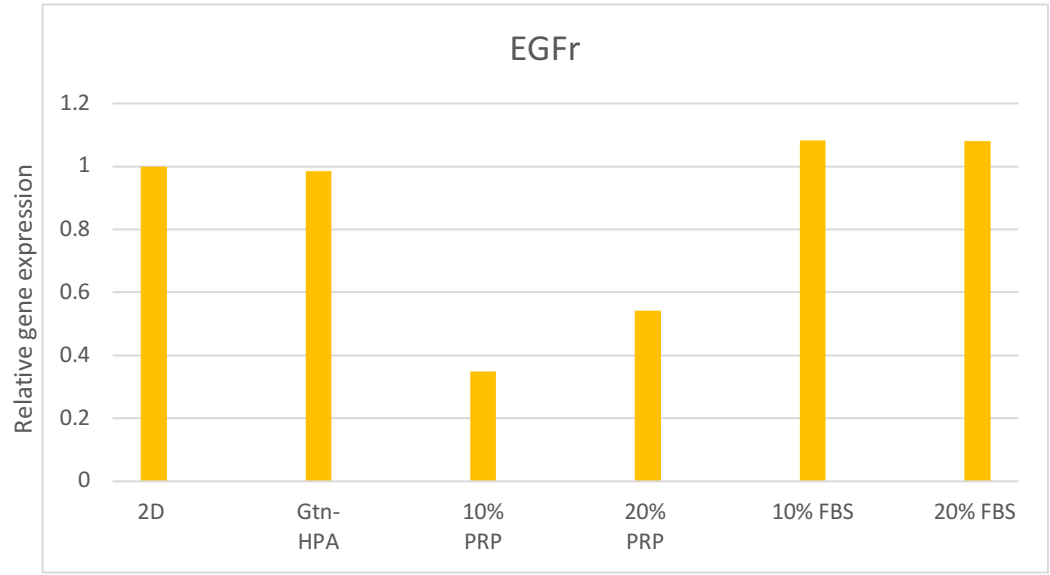

Fig. 26. Gene expression level of EGFr, donor #3.

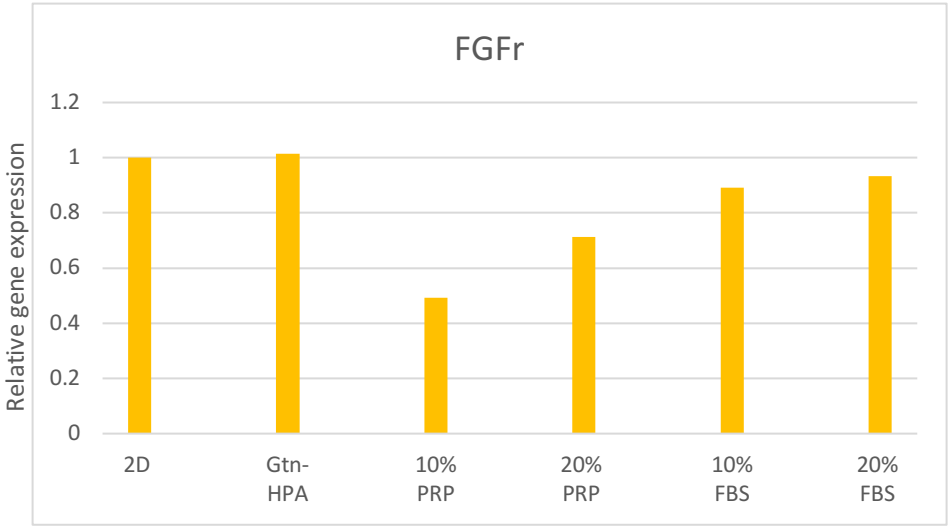

Fig. 27. Gene expression level of EGFr, donor #3.

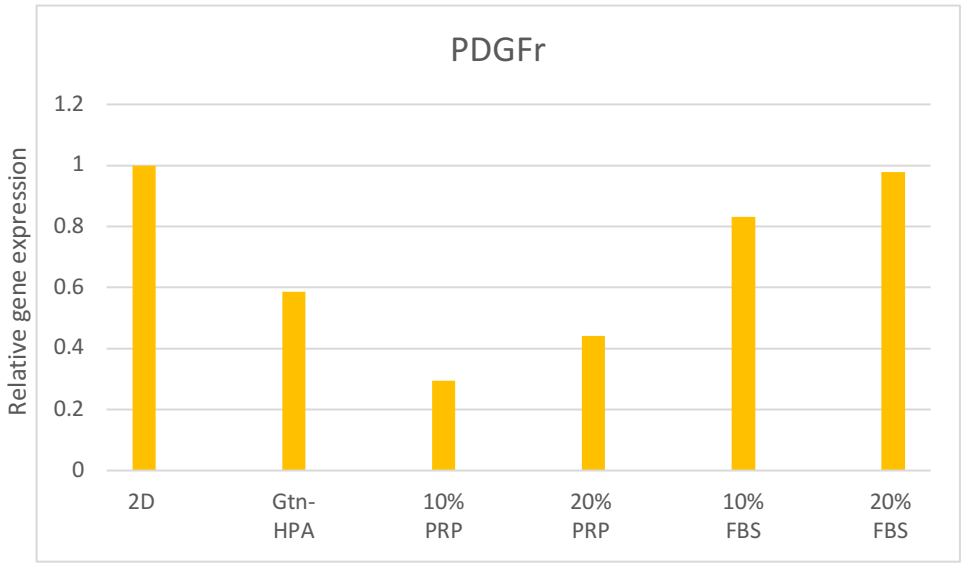

Fig. 28. Gene expression level of PDGFr, donor #3.

DISCUSSION

Treating periodontal defects has been a challenging task for periodontists. Platelet rich plasma has been used for various tissue engineering applications such as bone, cartilage, skin, and soft tissue (24). In clinical cases involving periodontal tissue defects, PRP has been utilized as a viable, safe and affordable option to enhance the regenerative potential of hard and soft tissue reconstruction surgeries (43, 44, 48, 53, 54). The clinical applications seem to be endless, however the in vitro and in vivo results are not always favoring the use of PRP for periodontal regeneration (39, 45, 55, 56).

The conflicting results seen in PRP studies can be attributed to the lack of standardized PRP preparation methods and platelet content variation between PRP donors (32, 57).

In our study, the platelet content of donors varied significantly both in whole blood and PRP. Having a patient with a low platelet profile at baseline will not produce effective PRP levels capable of expressing genes and producing proteins in sustainable concentration that allows for improved regenerative outcomes. Mazzoca et al, 2012 reported variable concentration of blood components as well as platelet counts after repetitive blood draws from same donors as well as seen in three different PRP separation methods (2 single-spin and 1 double-spin) (57). Based on the gene expression

results of this study, there was a higher trend favoring osteogenesis in patients with high concentration of platelets in the PRP. Therefore, we propose to standardize PRP protocols whereas the platelet counts of donors should be measured prior to clinical procedures to normalize the number of platelets per ml of PRP. Current protocols need to be adjusted to allow individual plasma concentrations to obtain optimal PRP levels. This is currently not seen as a common practice neither in clinical application or in in vitro and in vivo studies.

Weibrich et al, 2002 reported that donor age and sex have no influence in platelet counts and growth factor concentrations (58). On the other hand, Vavken et al, 2010 showed decreases in the expression of growth factor receptors TGF-B and FGF released by PRP with increasing donor age, but platelet count was not evaluated (59). In this study, there is a trend of upregulated gene expression of growth factor receptors in the 10% and 20% PRP groups for donors #1 and #5. Donor #7 growth factor receptor expression downregulation may be related to low PRP platelet levels but age could have played a role in this skewed result (age not presented here), therefore worth to be investigated.

The PRP protocol used in this study was previously described by Intini et al, 2009 and has been a common practice for many years at HSDM (35). The 2 centrifugation steps have specific spinning time and centrifuge specifications. Our protocol requires 2 spins, one for 2 minutes and 15 seconds and a second spin for 5 minutes at 1000g. Kobayashi et al, 2017 protocol is first spin for 5 minutes and second spin for 10 minutes at 1200g (56). As we can observe, studies have reported a variety of protocols and therefore comparing

results between different PRP centrifugation protocols is a challenge. As described by Ehrenfest et al 2014, each centrifuge has its own rotational speed, and this leads to significant differences in the platelet concentrate final product (60). Mazzocca et al, 2012 reported 3 different PRP protocols and they found higher platelet counts in PRP prepared by a single step centrifugation (5 minutes at 1500 rpm or 15 minutes at 3200 rpm) versus double-step centrifugation method (5 minutes at 1500rpm + 20 minutes at 6300 rpm) (57). Ultimately, the centrifugation protocol should also be standardized to allow consistency between PRP procedures.

Lastly, PRP needs to be activated to allow the release of growth factors from the platelets' alpha-granules. In this study, we utilized a double-activation method proposed by Marx et al, 1998 and reported in many studies (48, 53, 61). The use of calcium sulfate as solo activator has also been investigated (35, 54). We have proposed the use of the 2-activation method with thrombin and calcium chloride in this study since different activation methods was revised by Lacoste et al, 2003 and they reported that the double activation resulted in greater growth factor release (37).

Growth factors do not work alone, they function in a vast network of other growth factors, signals and cell-to-cell communication (24). Therefore, using PRP seems to be an optimal option to allow the release of multiple autologous growth factors to work in conjunction rather than a single recombinant growth factor application such as rhPDGF-BB or rh-BMP-2.

According to Kobayashi et al 2016 and 2017, the concentration of PDGF release of PRP at 15 minutes is statistically greater than observed in other time points, between 15 minutes to 10 days. At later time points, the total growth factor releases increase, but individual growth factor concentration is diminished (56, 62). This means that our protein quantification results at 15 minutes reveal the maximum concentration of growth factor that the PRP lysate is able to immediately release when incorporated to the Gtn-HPA. These results corroborate with previous studies showing that PRP releases PDGF and TGF-B in greater amount compared to other growth factors and cytokines. PDGF is known to be a powerful mitogenic (stimulates cell division) and chemotactic (cell recruiter) growth factor. TGF-B is also a mitogenic, angiogenic (stimulates blood vessels formation), and morphogenic agent (stimulates matrix production and guides osteogenesis) (27, 39). Thus, having our PRP incorporated to the hydrogel did not affect the growth factors release of these proteins and therefore they can execute their functions optimizing regeneration.

The injectable biopolymer used in this study presented to be a viable carrier for PRP. The Gtn-HPA incorporated to 10% and 20% PRP was permissive to maintain the viability of the osteoprogenitor cells, confirming that in this in vitro model the PRP is fully compatible when incorporated into the hydrogel. Using injectable hydrogels presents many advantages such as minimal invasiveness, ease of application and void-filling property (63). The capacity of cross-linking in situ is a huge advantage of the Gtn-HPA, whereas it can be injected in a liquid state and gelate into the defect within minutes by controlling the

gelation rate and stiffness with HRP and H_2O_2, respectively. Reduced gelation rate makes the protocol more applicable and stiffness is of major importance when applying carrier/scaffolds into areas that undergo forces, such as seen in periodontal defects. Thus, the injectable Gtn-HPA may be a promising PRP carrier for small periodontal defects, being delivered in the same manner as seen by local antibiotics.

The gene expression of osteoprogenitor genes and growth factor receptors presented a higher trend in the 10% and 20% PRP groups for donor #1 and #5 when compared to control groups. However, a significant difference was seen between donors and this can be related to the PRP platelet counts. As observed, donor #7 had 480 x 10^3 platelets/ml in PRP and presented gene expression in much lower levels for the 10% and 20% groups than when compared to donor # 5, which presented the highest concentration of platelets in the PRP lysate (1,140 x 10^3 platelets/ml). Therefore, even though the PRP protocol utilized was the same for all donors, the initial platelet count interferes in the final PRP levels leading to variable gene expression profile and growth factor release among different donors.

Taking in consideration the limitations of this study, specially related to small sample size and donor variability, but also considering the advantages of the osteogenic potential of PRP, added to the advantages of the reported PRP protocol, plus the advantages of the hydrogel properties herein used, we can conclude that the novel injectable Gtn-HPA

is a suitable carrier for PRP: cell viability is preserved, gene expression seems to favor osteogenesis and growth factor release is optimal.

CONCLUSION

This study reports, for the first time, the use of a novel injectable hydrogel incorporating PRP as a promising injectable scaffold for periodontal regeneration. Our results reveal the Gtn-HPA as a suitable carrier/scaffold for PRP with optimal growth factor release and enhanced gene expression favoring osteogenesis.

LIMITATION AND FUTURE STUDIES

Limitations:

In the present study, we were limited by a low sample size added to the high inter-variability between them which did not allow to conduct proper statistical analyses of our results. Thus, it is empirical that we augment the number of patients in order to obtain a higher *n* and greater power to our study.

Future studies:

- Increase the sample size for both protein quantification and gene expression experiments to allow statistical analysis;
- Incorporate PRP to another scaffold to compare to the efficacy of the Gtn-HPA;
- For protein quantification, add a control groups: PDGF or BMP-2, serving as growth factor positive control;
- Investigate the growth factor release of PRP incorporated into the Gtn-HPA in different time periods: 15 minutes - 10 days;
- Investigate the regenerative potential of the injectable Gtn-HPA hydrogel incorporating PRP in animal models, via histology, immunohistochemistry and micro-computerized tomography (micro-ct) analyses.

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