The Characterization and Quantification of Bioactive Components in Human Blood Clots for Tissue Healing and Engineering

Amanda Doodlesack | 2014
Biology | ScB

Brown University

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A thesis is submitted in partial fulfillment for the degree of Bachelor of Science with Honors in Biology

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BACKGROUND

Blood Clots

The human body's healing process is an incredible complex system of signaling pathways. Its purpose is to rid the body of foreign matter and diseased tissue and to regenerate new, healthy cells. Proper healing is an essential function of the body without which humans could not survive. Central to the healing process are blood clots, the platelets they entrap, and the growth factors derived from platelets.

There is a long history of the use of blood clots as an adjuvant in wound healing. The first to discover this use of blood clots was William Halsted during the 1890s. Halsted, a surgeon and founding professor at Johns Hopkins, realized that he could use blood clots to fill dead spaces in wounds in order to stop bleeding as well as to prevent bacterial infection.[1] Ever since, the popularity of the clinical use of clots and other blood-derived products in healing has risen and fallen in cycles.[2]

The role of blood clots in wound healing is multifold. First and foremost is the purpose of hemostasis. When the body is wounded, blood flow is perturbed, which initiates a clotting cascade. Through a process called coagulation, a clot develops consisting of cross-linked fibrin and other extracellular matrix components as well as platelets.[3,4] This three-dimensional clot functions as a sealant between the internal circulation and the outside environment, preventing both blood loss and invasion of infectious microorganisms. In addition to mediating hemostasis, the platelets entrapped within the matrix of the clot contain alpha granules, which are rich in growth factors, particularly platelet-derived growth factor (PDGF) and transforming growth factor (TGF). These growth factors, also known as cytokines, induce the wound-healing cascade by recruiting inflammatory cells, immune cells, and repair cells to the site of injury.[5] In addition to

PDGF and TGF, other growth factors including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are also derived from platelets and found in blood clots. These growth factors have been implicated as cell signaling molecules critical to the tissue repair process.[4]

Given the presence of these critical growth factors in blood clots that form naturally in healing, the hypothesis that blood clots could be applied clinically as an adjuvant in tissue healing is a logical step.

PRP

An alternative source of growth factors to aid in healing is platelet rich plasma (PRP). PRP has been used clinically in humans since the 1970s for its ability to enhance healing due to its high concentration of autologous growth factors.[5] These growth factors are derived from platelets, just as those found in clots. Platelet rich plasma is defined as plasma, the fluid portion of the blood, and platelets, small non-nucleated bodies in the peripheral blood that serve mainly hemostasis. While normal platelet counts in whole blood range from 150,000/μL to 350,000/μL, the concentration of platelets in platelet rich plasma is at least 1,000,000/μL suspended in plasma, up to a six- to seven-fold increase.[5,6] Accompanying this increase in platelet content is a three to five-fold increase in growth factor concentration. Growth factors found in PRP are derived from platelets, and therefore are the same as those found in natural clots, mainly PDGF, TGF, VEGF, and FGF.[5]

Platelet rich plasma is made from anticoagulated blood to prevent clotting. Without anticoagulants, platelets would become part of the clot that is formed and therefore would not be concentrated in the plasma. To prepare PRP, citrate is added to whole blood to bind to the

ionized calcium, which inhibits initiation of the clotting cascade. The anticoagulated blood is then centrifuged in two steps, first to separate red and white cells from the plasma and platelets, and secondly to separate PRP from platelet poor plasma.[5]

To maximize delivery to the target site in clinical use the PRP must be artificially clotted. Clotting is initiated by adding either bovine thrombin, adding calcium chloride to initiate formation of autogenous thrombin, or adding type I collagen to activate platelets directly.[5] Clotting initiation also activates platelets and their growth factors, activating the healing properties of PRP.

High levels of PDGF, TGF, VEGF, and FGF, known to have an essential role in cell proliferation, chemotaxis, cell differentiation, and angiogenesis have piqued clinical interest in PRP as a delivery medium of growth factors to enhance healing. Initially, PRP was recognized for its efficacy in bone and tissue repair in dentistry and maxillofacial surgery. Its applicability has spread, and recently has risen in the field of orthopaedic surgery. PRP has shown potential in healing bone, muscle, ligaments, and tendons, and has been reported to improved post-surgical recovery.[7] However, in order to further advance clinical applications of PRP, it must be better characterized and understood.

ClotMaster™

A novel device developed by Pierce Surgical Instruments in Waterbury, VT, the ClotMaster™ Hula Cup provides an alternative method to produce a platelet rich, blood derived healing adjuvant in solid form. The ClotMaster™ Hula Cup uses autologous whole blood to produce a wild-type clot, rich in platelets and encapsulating the same growth factors found in clots that form naturally and in PRP. The production method for ClotMaster™ is much simpler

than for PRP. By swirling a sample of whole blood in the Hula Cup for no more than 10 minutes, a wild type clot rich in platelets and growth factors is formed. This solid clot can be removed from the Hula Cup and applied to the site of a surgical or traumatic wound as a healing adjuvant, reminiscent of William Halsted's discover over a century ago.

The ClotMaster™ device consists of a sterile 140 mL polyethylene cup, lid, and sintered glass rod down the center. [Figure 1] The height of the glass rod can be adjusted according to manufacturer's specifications to produce different types of clots.

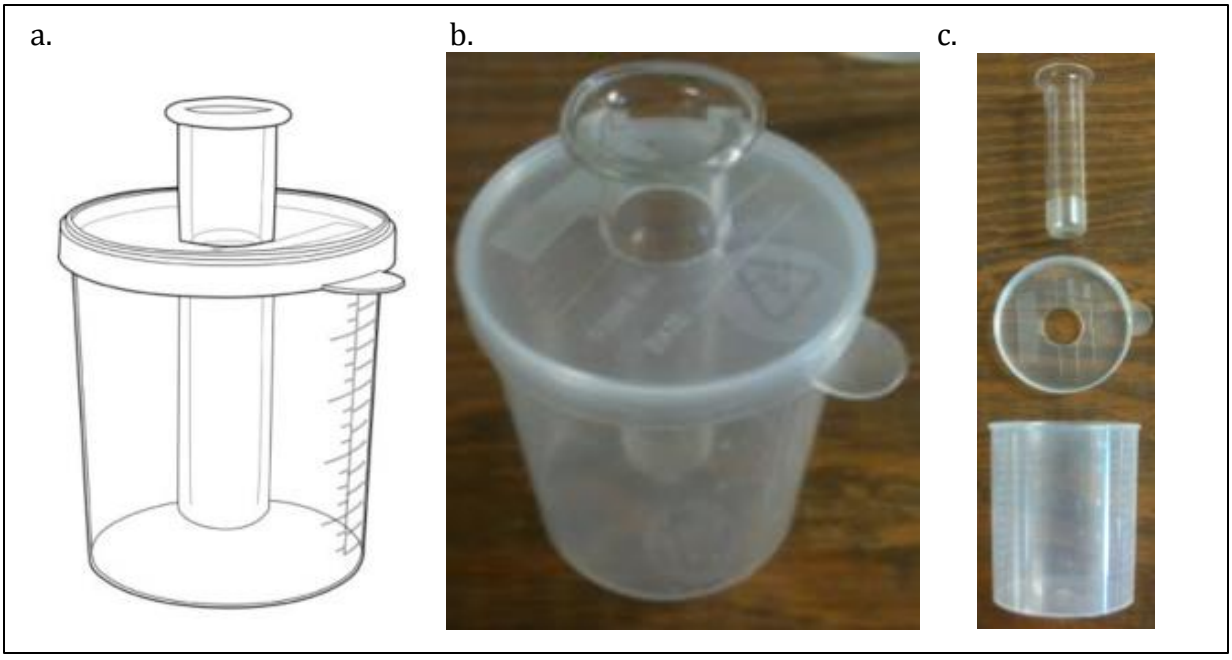


Figure 1. The ClotMaster™ Hula Cup Device: a. ClotMaster™ prototype, **b.** ClotMaster™ Hula Cup device assemble, **c.** ClotMaster™ device components dissasembled

Project Aim

The goal of this project is to assess the viability of clots produced using ClotMaster™ as tissue healing adjuvants by comparing the bioactive components of such clots to the bioactive components of PRP. By conducting extensive literature research on platelet rich plasma, its preparation methods, its role in tissue healing, and its shortcomings, parameters by which to

evaluate ClotMaster™ were established. ClotMaster™ clots were then evaluated based on these metrics. Based on the results of these studies, the potential for ClotMaster™ to serve as a better alternative to PRP for clinical use was assessed.

INTRODUCTION

Although platelet-rich plasma has been utilized clinically for two decades, there still lacks a consensus on the content, efficacy, and consistency of PRP. Several definitions have been proposed for PRP; as of now the only clear definition is based on the number of platelets captured. One definition of PRP is a fraction of autologous whole blood with a platelet concentration of at least 1-million platelets/μL. Alternately, others define PRP as any platelet concentration above physiological baseline, typically ranging from 150,000 /μL to 350,000/μL.[6] Notably missing from these definitions is any description of growth factor content, which many studies have shown to be crucial to PRP's healing properties.

A major reason why PRP is so vaguely defined is that its contents are highly inconsistent. There are numerous preparation systems commercially available, each using slightly different preparation techniques, and each producing a different product. In addition, because PRP is prepared from the patient's whole blood, even when using the same system the product can vary from patient to patient. Genetic, dietary, and environmental factors all influence a person's blood content, and therefore can alter the content of PRP produced.

The first metric of PRP on which different production methods vary widely is platelet capture rate. Platelet capture rate is thought to be an important measure of healing capacity because platelets are the first to arrive at the site of tissue injury and have the potential to release essential growth factors which mediate healing.[6] There are about 20 major commercial PRP preparation systems currently available. Platelet capture efficiency rates range from a meager 17% in Vivostat, up to 70-80% in DePuy, Symphony II.[6] Each of these systems varies in centrifugation time and force, anticoagulant, required blood volume, and final PRP volume produced.[7] All of these factors contribute to the highly variable platelet capture rates.

Another aspect of PRP preparation that varies widely across commercial systems is the activation procedures. Activation refers to the degranulation of α-granules in platelets to release growth factors and the activation of fibrinogen to fibrin in order to initiate the formation of the clot matrix.[6] A few different major methods of activation are employed by PRP preparation systems: addition of bovine thrombin, addition of calcium chloride, freeze/thaw cycles, and direct exposure to collagen in vivo.

Several systems add bovine thrombin to activate the clotting mechanism in PRP and to activate platelets. However, because bovine thrombin is xenogeneic, it poses the risk of an immunogenic response. In addition, activation via bovine thrombin results in release of growth factors from α-granules very quickly. About 70% of the stored growth factors are released within 10 minutes, and nearly all of the growth factors are released within an hour. After that, only small quantities of growth factors may continue to be released by the platelets for the remainder of their 8 to 10 day lifespan.[5] This is a rapid, high concentration release of growth factors into the wound site that may not lead to optimal healing.

Addition of $CaCl_2$ is used to initiate the formation of autogenous thrombin from prothrombin, which then activates the clotting cascade. This results in production of a clot matrix, and minimizes platelets activation so that platelets release growth factors more slowly over a 7-day period.[4] This more gradual delivery of growth factors may better resemble natural healing and therefore lead to enhanced results over bovine thrombin activation.

Another method of activation is to use freeze/thaw cycles to damage the platelets, thereby causing the cells to lyse, degranulate, and release growth factors from the α-granules. As of now there is no precise consensus on the number of freeze/thaw cycles required for optimal results.

While this is a useful activation method for in vitro study of PRP because it does not chemically alter the content, due to the extensive time required, it is not practical for clinical purposes.[6]

One last method of PRP activation is to depend on exposure to autologous collagen I fibers present in the wound site to activate the PRP in vivo. Non-activated liquid PRP is applied to the wound site, and allowed to activate naturally upon contact with collagen. In vivo activation is the preferred method for activation in clinical use because it does not require any exogenous activation factors and results in a sustained release of growth factors. There is no risk of immunogenic reactions because no foreign material is introduced.[6] However, clinicians have less control over this method, and there is likely to be more variation by wound site, as each patient and each wound has a varying amount of collagen I available for activation.

Not only do PRP products vary in activation method and platelet capture efficiency, but they also vary in the concentration of growth factors present. In a 2006 study conducted by G.C. Leitner et.al., PDGF-αβ content was measured using immunoassays in four different PRP production systems as a function of time following activation. While growth factor content increased across all four systems over the 72 hours following activation, the total amount varied considerably. The lowest yield system had a maximum PDGF- αβ content of about 5000 μg/mL, whereas the highest yield system reached a maximum 250,000 μg/mL.[8] The amount of PDGF- αβ released was found in the study to correlate with the platelet capture of each system. The higher the platelet capture rate, the greater the concentration of growth factors. This is a further indication that higher platelet capture rate increases bioactivity. Another study conducted by Castillo et. al. in 2011 compared levels of PDGF- αβ, PDGF-ββ, TGF-β1, and VEGF using ELISA across three different PRP preparation systems. Results were again inconsistent. Levels of PDGF- αβ varied from 9.7 ng/mL to 34.4 ng/mL, PDGF-ββ ranged from 14.8 ng/mL to 33.0

ng/mL, TGF-β1 ranged from 0.1 ng/mL to 0.2 ng/mL and VEGF ranged from 0.3 ng/mL to 1.2 ng/mL.[7] This incredible variability clearly demonstrates that not all PRP is the same.

Due to the variability of the content of PRP, clinical outcomes inevitably also vary greatly. There are several studies published claiming excellent outcomes, but these studies are limited. Many are based on a limited series of case reports, which may or may not have controls and all involve small sample sizes. Additionally, studies do not utilize standardized dosing because each PRP preparation is different. Therefore, it is nearly impossible to productively analyze the results and determine any significant findings. In order to learn more about the true effects of PRP in tissue healing, PRP content must be standardized, and a randomized control study must be conducted.

That being said, the evidence for success in clinical applications cannot be dismissed. The number of case report studies reporting success rates of 60%, 70%, and even 99% is substantial. These studies span a wide variety of clinical fields as well. Case reports demonstrate success in treating chronic tendinopathy, bone healing, acute ligamentous injuries, acute muscle injuries, and intraoperative use.[5] Use of PRP clinically is also reported to be fairly straightforward for clinicians. Although each system varies in its centrifugation and activation process, all are comparable. Blood is first drawn from the patient (amount varies from 30 mL to 60 mL depending on the system). The blood is then centrifuged for anywhere from 5 minutes to 15 minutes, and then PRP is separated out and activated. The entire preparation process takes anywhere from 15-30 minutes, and depends not only on the manufacturer's protocol but also n the clinician carrying out the procedure.[9] This time required to prepare PRP could be problematic, as increasing the time a patient is open in the operating room increases the likelihood of

complications and infection. Also problematic is the high cost of PRP prep systems. Commercial systems cost anywhere from $5,000 to $16,000.[10]

Despite its shortcomings, PRP is widely applicable and is worthy of further investigation and development. It is considered the current gold standard of autologous blood derived healing adjuvants for clinical use. Research to better understand flaws in PRP, its preparation, and its application and efforts to develop an optimized adjuvant based on the same concepts as PRP could lead to serious advancement in tissue healing.

METHODS

In order to assess the healing capacity of ClotMaster™ clots and compare them to PRP, parameters for measuring healing enhancement were established. These parameters were based on thorough literature review of studies characterizing the bioactive content of PRP and comparing products of different PRP commercial preparation systems. The majority of studies were found to measure PRP and compare PRP products based primarily of growth factor content and platelet capture rate. The key growth factors commonly measured were PDGF-ββ, TGF-β1, VEGF, and FGF2. These growth factors are logical measures of healing capacity, as they, along with a few other growth factors, play crucial roles in the tissue-healing cascade. [Figure 2]

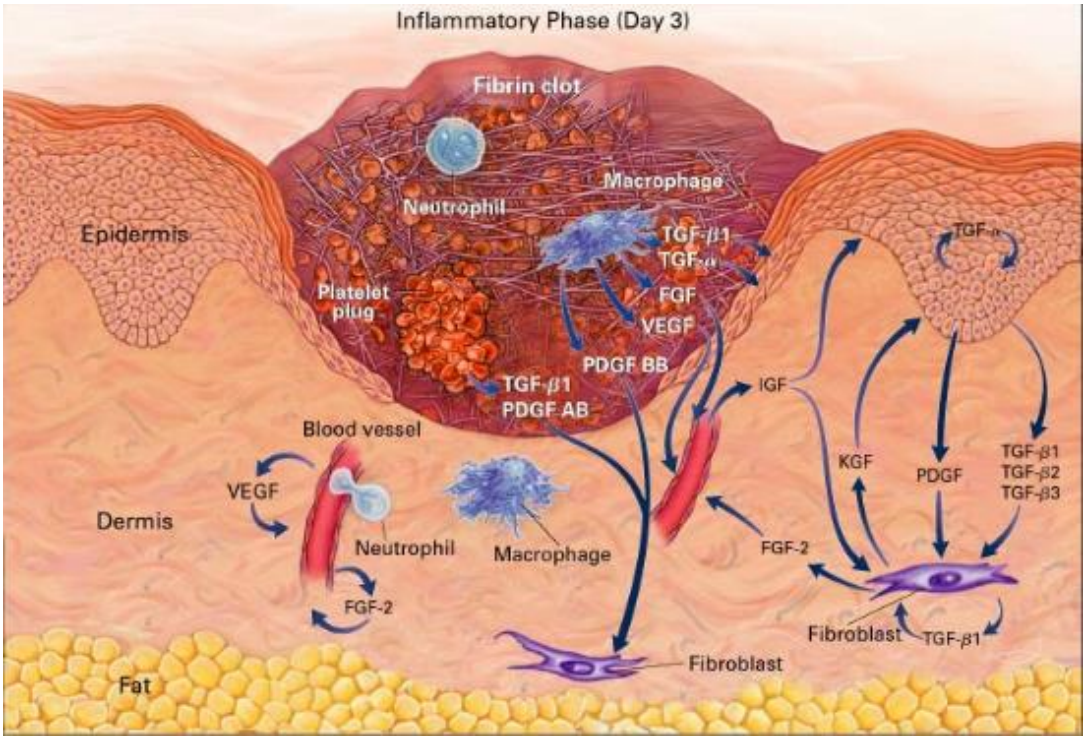


Figure 2. A Cutaneous Wound Three Days after Injury.
Growth factors thought to be necessary for cell movement into the wound are shown. TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor β1, β2, and β3, respectively ; TGF-α transforming growth factor α; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF-αβ, and PDGF ββ platelet-derived growth factor, platelet-derived growth factor αβ, and platelet-derived growth factor ββ, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth fact **[http://www.nejm.org/doi/full/10.1056/NEJM199909023411006]**

Based on extensive literature research, the parameters by which to compare ClotMaster™ clots to PRP were chosen to be platelet capture rate and presence of PDGF-ββ, TGF-β1, VEGF, and FGF.

Comparison of Platelet Capture Rate

Platelet capture rates for PRP were obtained from literature. Several studies measured platelet capture rates across different preparation systems using different methods. The most comprehensive information was found in a review paper by Wasterlain et. al. in 2012, which compared platelet capture rates for 17 commercial PRP preparation systems. Capture rate data was obtained from manufacturer's literature, and was reported as a percentage. This percentage gives the percent of platelets from whole blood that is captured in the PRP portion that is separated through the preparation process.

To obtain platelet capture rates for ClotMaster™ clots, an indirect measuring method was used. Platelet capture efficiency was calculated by measuring platelet content in a sample of whole blood both pre- and post- clot formation. The difference in platelet count pre- and post-clot formation gives the count of platelets captured in the clot. Platelet capture rates were measured for gel clots only.

35 mL of whole blood was obtained through venipuncture from 10 healthy volunteers, free of clotting abnormalities and with no recent use of non-steroidal anti-inflammatory medication (NSAIDs). 5 mL of blood was set-aside in standard blood collection tubes for complete blood count (CBC), which includes platelet count. The 30 mL remaining was placed into ClotMaster™ Hula Cup for clot formation. The Hula Cup was swirled in a circular fashion for 10 minutes, according to manufacturer's specifications, and a clot was formed. This clot was removed from the container, leaving behind blood plasma not included in the clot. This

remaining plasma was removed and put into a 5 mL blood collection tube also for CBC evaluation. Platelet counts from the CBCs done on pre-clot formation whole blood and post-clot plasma were compared. The difference in platelet count between pre-clot and post-clot CBC gives the platelet count of the solid clot that formed. This platelet count was then divided by the baseline platelet count given by the initial whole blood CBC to get a percentage. The values obtained for each of the 10 individuals were averaged.[11]

Platelet capture rates for ClotMaster™, as percentages, were compared to the PRP preparation systems Arteriocyte Magellan, Biomet GPS, Harvest SmartPReP2, and MTF Cascade. These four commercial systems were chosen for comparison because they are regarded in the literature as the top performing and most commonly used systems in the clinic, and are therefore the gold standard products and the competition for ClotMaster™.

Growth Factor Content

ClotMaster™ Hula Cups are able to produce two different types of clots: gel clot and dense fibrous clot. Positioning of the sintered glass rod and timing of HulaCup swirling are what determine which type of clot is formed. Both the gel clot and dense fibrous clot were produced, analyzed, and compared to PRP in this study.

Gel Clot Formation:

To produce a gel clot, 35 mL of peripheral blood was drawn using a butterfly needle into a sterile syringe. It was important that the blood was drawn slowly so as to prevent excessive turbulence in the liquid that might induce early clotting. Once drawn, the 35 mL of whole blood was immediately transferred slowly and carefully into the Hula Cup, again trying to avoid turbulence. The sintered glass rod was positioned following the manufacturer's specifications for

a gel clot so that there was approximately 6 mm of space (manufacturer's protocol specifies >5 mm) between the base of the Hula Cup and the end of the rod [Figure 3]. The lid of the Hula Cup was close with the rod in position, and the cup was swirled by hand in a circular fashion for exactly 1 minute. After 1 minute of swirling, the Hula Cup was set down and allowed to stand for 9 minutes. After this procedure, a semisolid gel clot was formed.

Figure 3. Hula Cup set up for gel clot.
Sintered glass rod is positioned ~6mm from base of Hula Cup.

Dense Fibrous Clot Formation:

To produce a dense fibrous clot, again 35 mL of peripheral blood was drawn using a butterfly needle into a sterile syringe. The blood was immediately transferred carefully into the Hula Cup. For a dense clot, manufacturer specs dictated that the sintered rod be positioned all the way down so that it made physical contact with the base of the Hula Cup [Figure 4]. There was no space left between the end of the glass rod and the base of the Hula Cup. The lid was closed, and the hula cup was swirled continuously by hand in a circular fashion for 10 minutes. After 10 minutes of nonstop swirling, a dense fibrous clot was formed around the perimeter of the glass rod, forming an annulus.

Figure 4. Hula Cup setup for dense fibrous clot.
Sintered class rod is all the way down, making contact with the base of the cup.

Morphology

Gross physical characteristics of the clots were observed visually and tactilely. Clots were examined for color, shaped, sheen, malleability, ductility, and structural integrity. Forceps were used to remove the clots from the Hula Cups. Clots were then stretched, squeezed, and further manipulated by hand to get a basic sense of the physical properties.

Fixation, processing, and sectioning:

Once clots were formed, they were removed carefully from the hula cups and placed into a sterile 200mL vessel containing 190mL of 10% zinc formalin unbuffered fixative. Clots had adhered somewhat to the surface of the Hula Cup (gel clot) and sintered glass rod (dense fibrous clot). Using sterile forceps, clots were gently separated from the surfaces and transferred in one piece into the fixative. The clots were allowed to sit in fixative for 48 hours at room temperature. Once fixed, the clots were embedded in paraffin, then sectioned into 5μm thin cross sections, cut parallel to the orientation of the position of the Hula Cup base during clot formation.

Immunohistochemical Staining:

Sections of both the dense fibrous clot and the gel clot were stained for all six growth factors, PDGF-BB, TGF-B1, VEGF, and FGF2. Antibodies used for all growth factors were rabbit polyclonal antibodies obtained from Abcam. Antibodies were diluted according to manufacturer's protocols. Three dilutions spanning the recommended range were tested for each antibody, and the dilution that produced the best results visually was chosen. Antigen retrieval was done using pepsin, and secondary antibodies were supplied from a Vector Labs Vectastain ABC Rabbit IgG kit. Slides were then counterstained with hematoxylin. Negative controls were also prepared for both the dense fibrous clot and the gel clot for comparison.

Analysis:

Immunohistochemical staining was observed using a Nikon Eclipse E800 light microscope at 40x magnification. Sections were assessed for presence of positive staining compared to the negative control, and for patterns in the distribution of positive staining throughout the cross section of the clot. Abundance of positive staining for each growth factor was compared between the dense fibrous clot and the gel clot, and relative to the other growth factors examined.

RESULTS

Morphology

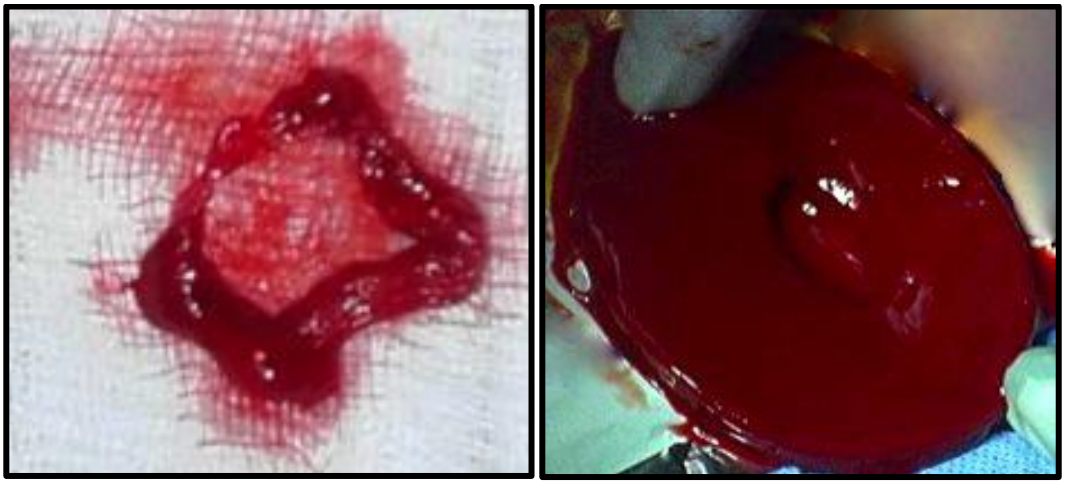


Figure 5. Dense Fibrous and Gel Clot, removed from Hula Cup.
Left: dense fibrous clot removed from Hula Cup and placed on gauze
Right: gel clot removed from Hula Cup and held between fingers

The color of both the gel and dense fibrous clots was a deep red, the color of oxygenated blood rich in RBCs. Clots were highly flexible, and are fairly robust. They were easily removed from the Hula Cups and manipulated by hand without damage.

The gel clots were slightly more delicate than the dense fibrous clots. They were highly flexible and appeared shiny and wet, similar to jelly. The top surface of the gel clots was perfectly smooth and lustrous. They were not able to withstand significant stretching without tearing, and therefore were not highly elastic. They were somewhat compressible when pressure was applied, and they returned quickly to their original form. The gel clots were malleable, and could be molded into different shapes permanently if desired.

The dense fibrous clots were more robust than the gel clots. They were less susceptible to damage during handling. They did not appear as shiny as the gel clots, and were more irregular in their shape. The dense clots formed an irregular annulus around the sintered glass rod inside the

Hula Cup, and retained the annulus shape following removal. They were ductile, able to be stretched considerably without damage [Figure 6]. The elongation was largely observed to be plastic deformation, as the clots did not return to their original length after release.

Figure 6. Dense Fibrous Clot elongated to ~6 cm.

Examination of the clot sections with H&E staining revealed clear presence of biconcave intact erythrocytes, or red blood cells [Figure 7]. RBCs were the most prevalent cell type in the clot. Scattered throughout the image there are cloudy, whitish patches that are slightly out of focus. Given the 3-dimensional nature of the section, albeit 5μm thick, these patches are likely in a different plane than the RBCs that are in focus. It is thought that these opaque whitish patches may be clusters of platelets agglutinating within the clot. Also clear in the image are white

spaces. This shows that the gel clot was somewhat porous. This demonstrates the potential for the clot to serve as a scaffold for tissue engineering as well as its function as a hospitable scaffold for natural infiltration of cells during healing.

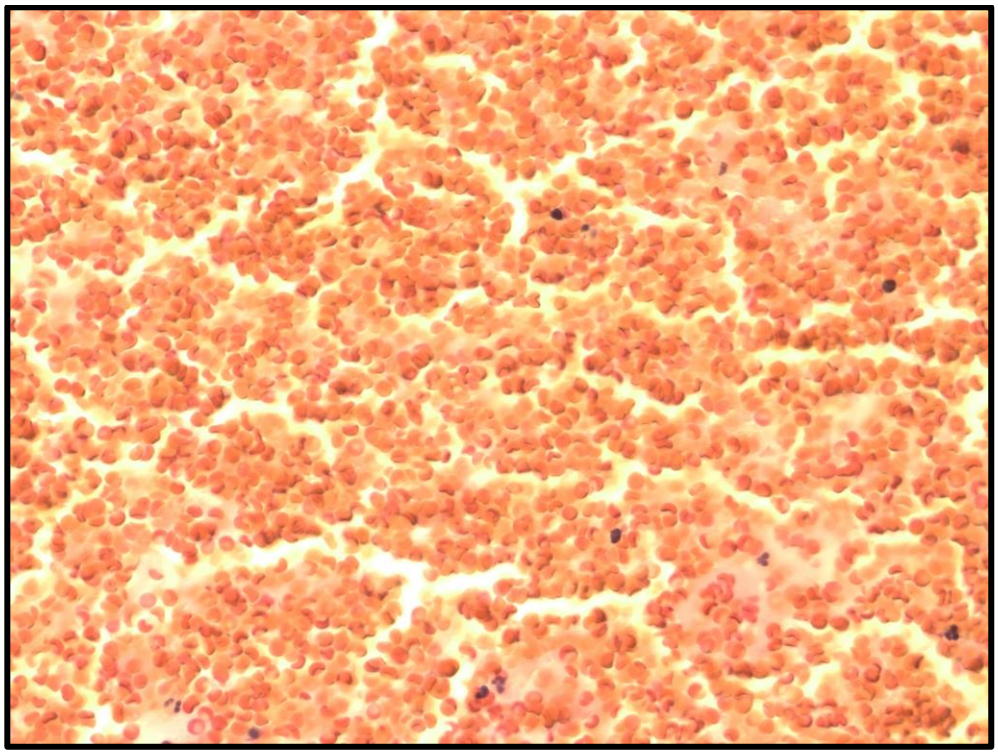


Figure 7. H&E staining of Gel Clot. Image was taken using a Nikon Eclipse E80 light microscope at 40x magnification. Section was 5μm thick.

Immunohistochemistry

Results of immunohistochemical staining indicate that TGF-β1, PDGF-ββ, VEGF, and FGF2, are present in human blood clots produced by the Hula Cup. This demonstrates that

ClotMaster™ clots deliver the same healing growth factors found in PRP, and therefore have the potential to enhance healing [Figures 8 and 9].

Gel Clot:

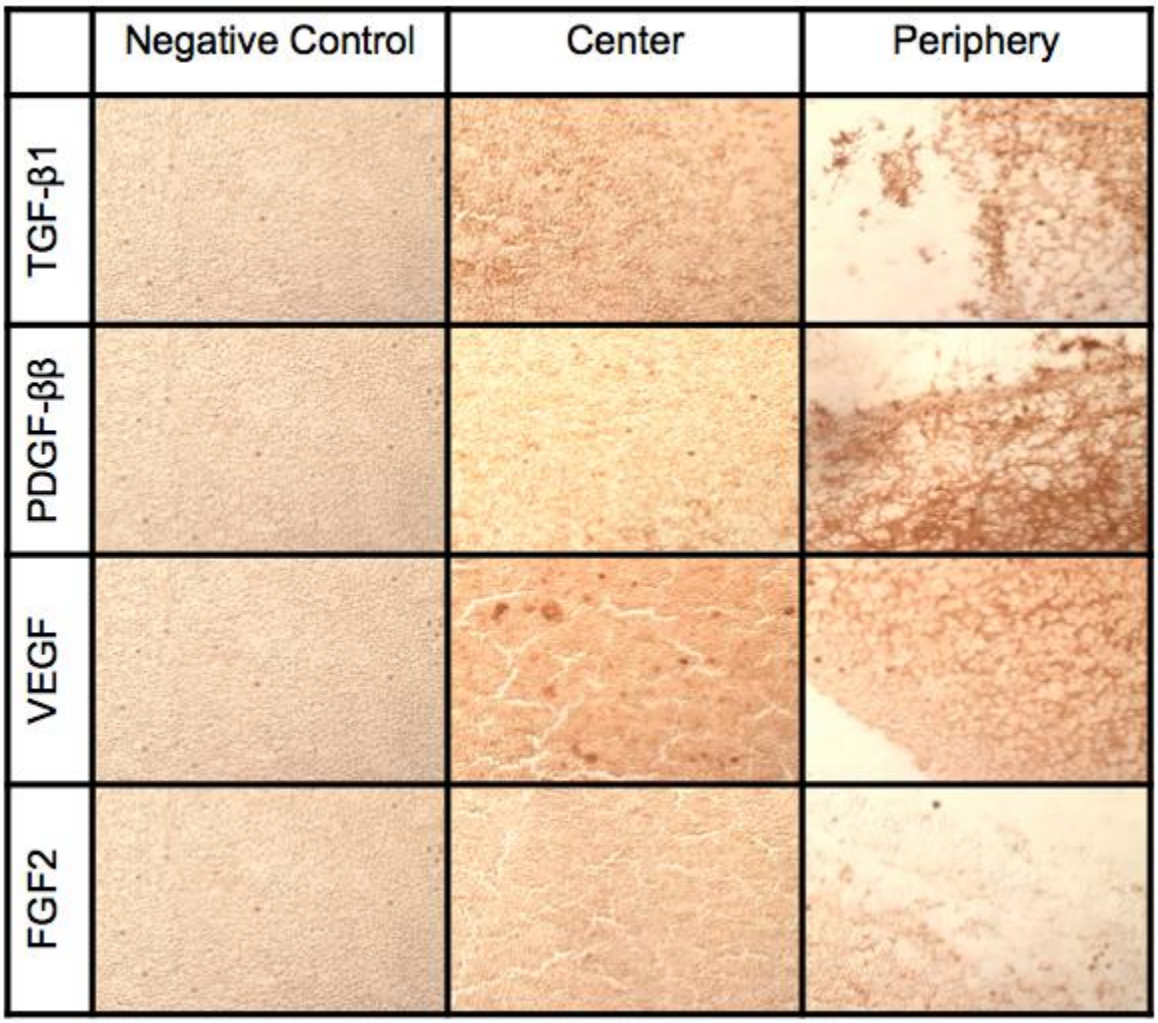


Figure 8. Immunohistochemical staining of gel clot sections. Sections were 5 μm thick. Four growth factors were examined. From top to bottom they were TGF-β1, PDGF-ββ, VEGF, and FGF2. The three columns from left to right represent negative controls, images of the center, and images of the periphery of the clot. Positive presence of growth factors is indicated by darker red/brown spots, indicating binding of antibodies. Images were obtained using a Nikon Eclipse E800 light microscope at 40x magnification. A higher intensity of staining was found at the periphery for each growth factor. VEGF and TGF-β1 had a greater distribution of positive staining, while FGF2 showed little positive staining.

Figure 8 depicts immunohistochemical staining of gel clot sections. Comparing the center column and right column to the left hand negative control, it is clear that there is positive staining for all four growth factors. Reddish brown staining indicates positive presence of growth factors, and there is clearly more red/brown staining in the right two columns than the left hand negative control. As shown by the right column, there is a greater staining intensity around the periphery of the blood clot, where the sample made physical contact with the Hula cup, as compared to the center of the clot depicted in the center column. This could be due to mechanically induced damage to the platelets that made contact with the outer perimeter of the Hula Cup. It is possible that turbulence along the walls of the Hula Cup led to degradation of the α-granules in peripheral platelets, causing them to release growth factors. The possibility that physical contact induced activation of TGF-β1, leading to a greater concentration of positive staining, was also considered. TGF-β1 is known to have a latent form that can be activated. However, literature research did not indicate any presence of a mechanoreceptor in the activation process of TGF-β1.

In addition, the intensity of TGF-β1 and VEGF staining in the clots is greater than PDGF-ββ and FGF2. This is consistent with results found in studies evaluating the presence of growth factors in PRP. The increased positive staining for VEGF and TGF-β1 may be because there is actually a greater presence of these two growth factors in the clots than VEGF and FGF2. This result could also be due to inconsistent staining or due to the inconsistent dilutions of antibodies during staining. In order to accurately compare the relative concentrations of growth factors in the clots, ELISA would be required. ELISA would also provide an absolute concentration of growth factors, which could be further compared to ELISA analysis of growth factors in studies on PRP.

Dense Fibrous Clot

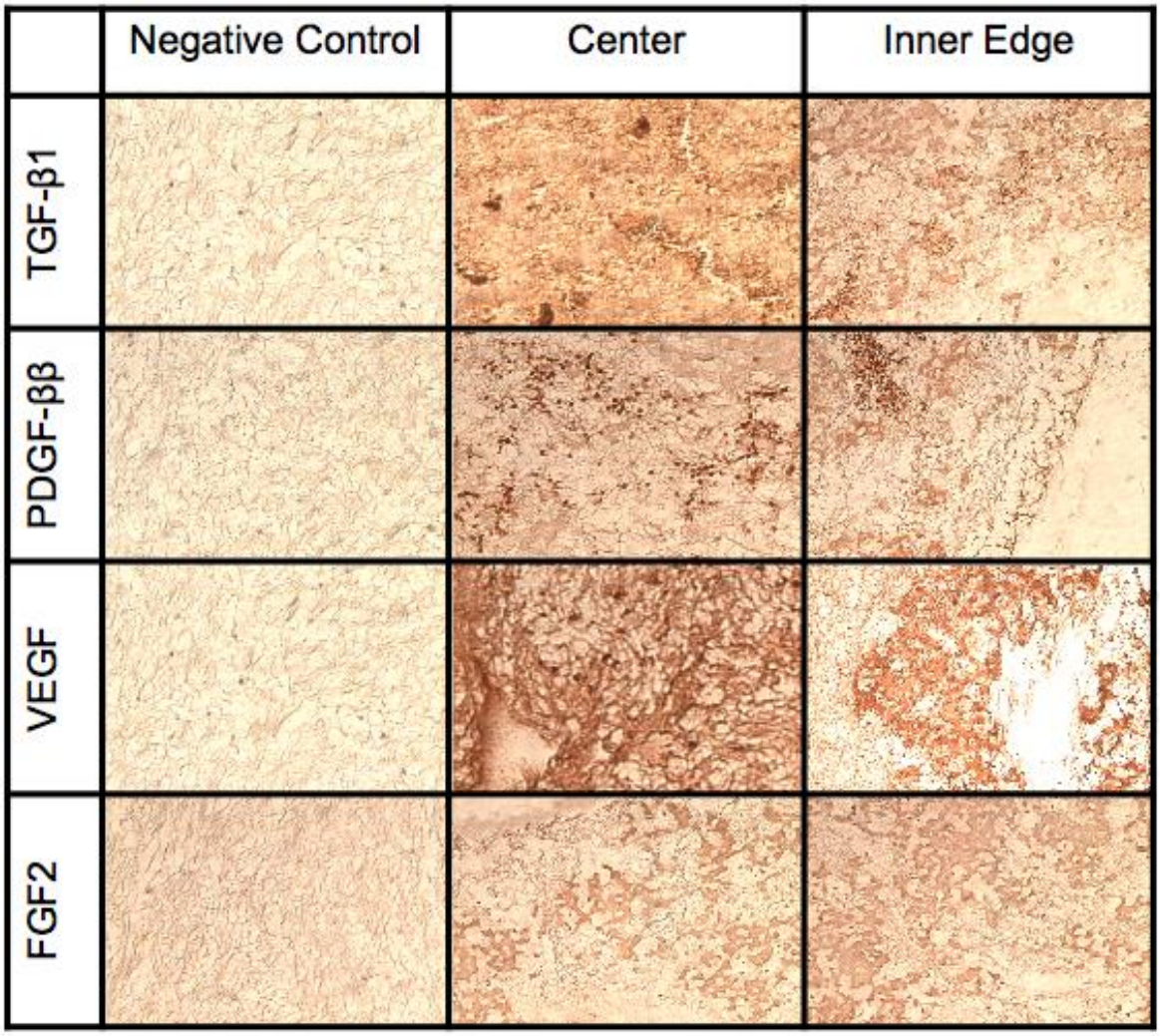


Figure 9. Immunohistochemical staining of dense fibrous clot sections. Sections were 5 μm thick. Four growth factors were examined. From top to bottom they were TGF-β1, PDGF-ββ, VEGF, and FGF2. The three columns from left to right represent negative controls, images of the center, and images of the inner edge of the clot that was attached to the sintered glass rod. Positive presence of growth factors is indicated by darker red/brown spots, indicating binding of antibodies. Images were obtained using a Nikon Eclipse E800 light microscope at 20x magnification. A higher intensity of staining was found at the periphery for each growth factor. VEGF and TGF-β1 had a greater distribution of positive staining, while FGF2 showed little positive staining.

Immumohistochemical staining of the dense fibrous clots showed similar results to staining of the gel clots. TGF-β1 and VEGF showed the greatest positive staining, while FGF2 showed the least positive staining. Because the dense fibrous clots form as an annulus around the

sintered glass rod, only the inner perimeter of the annulus makes contact with the glass surface. In looking at staining at the periphery, the inner perimeter was used. Unlike the gel clot, there was not a considerable difference in positive staining comparing the inner edge to the center of the annulus clot. This is likely because the width of the annulus (distance between the inner and outer radius) is much less than the diameter of a gel clot. Therefore, the middle of the of clot (halfway between the outer and inner radius of the annulus) is much closer to the periphery (inner radius), and so any potential growth factor activation promoted by physical contact with the sintered glass rod could extend into the center.

Once again, in order to precisely compare relative amounts of growth factors and absolute concentrations, ELISA is required. However, gross observation of positive staining in the dense clots appears to show a greater concentration of growth factors compared to the gel clot. This could be due to increased mechanically induced growth factor release, as a greater portion of the dense fibrous clot touches the sintered rod compared to the portion of the gel clot that touches the outer perimeter of the Hula Cup. Alternately, this could be due to an actual increase in platelet capture and growth factor capture. Further studies, including platelet capture rates for gel vs. dense fibrous clots as well as ELISA to quantify growth factor presence are required to better assess the difference between the content of the two clots.

Platelet Capture Rates

Platelet capture rates, or the percentage of platelets that are captured in PRP, varied widely across commercial prep systems. Capture rates ranged from as low as 17% in Vivostat PRF to as high as 70-80% in DePuy, Symphony II. These results further demonstrate the inconsistency in PRP product. The table below [Figure 6] shows platelet capture rates published by

manufacturer's literature obtained for eleven major commercial preparation systems. Other commercial systems are available, but platelet capture rates were not published.

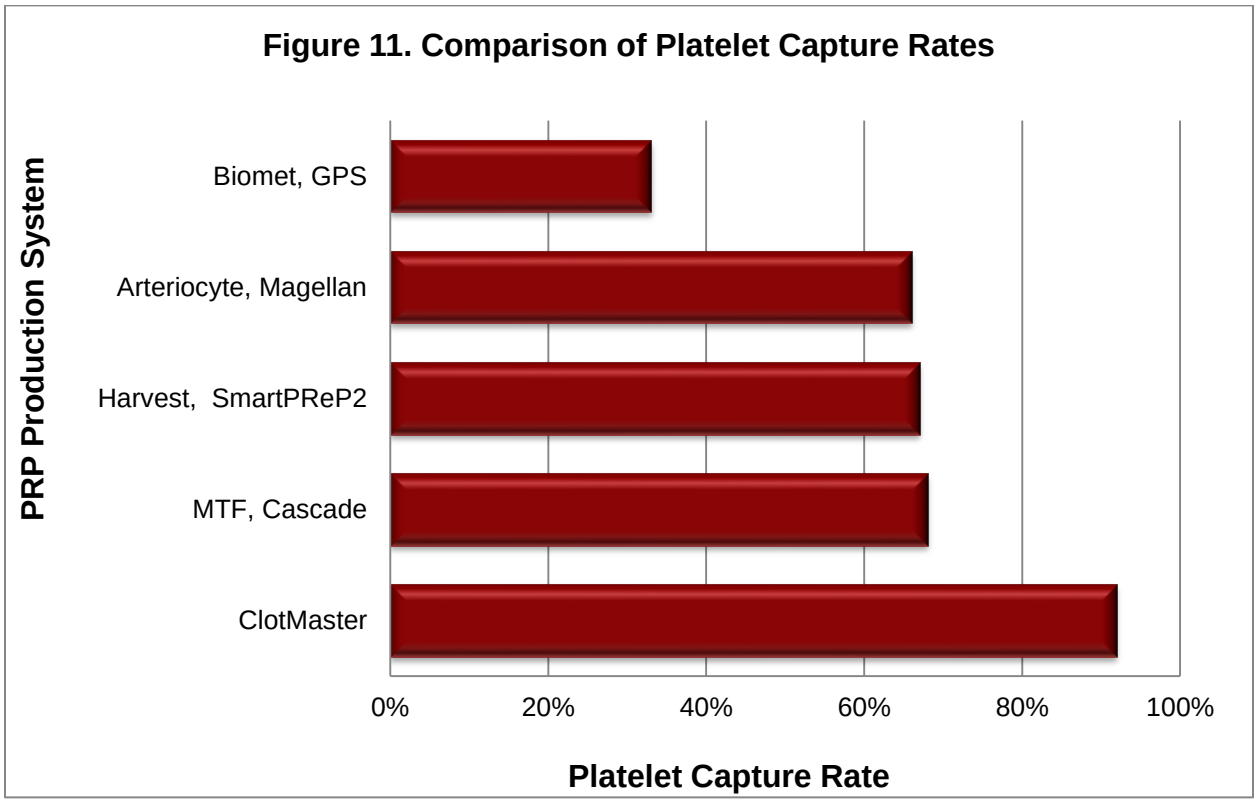
Figure **10**. Platelet Capture Rates for Commercial Systems[6]

System (company, product)	Capture Efficiency
Aesthetic factors, Selphyl (formerly FIBRENET)	66%
Arteriocyte, Magellan	66%
Biomet, GPS III	23-43%
DePuy, Symphony II	70-80%
Emcyte, Genesis	60-70%
Harvest, SmartPReP2	68%
MTF, Cascade	68%
PPAI, Secquire Cell Separator	31%
Stryker/regenLab, RegenKit-THT	65%
Vivostat, Vivostat PRF	17%
3i Implant Innovations	58-69%

Of the eleven systems shown in Figure 10, four were found in the literature to be used more commonly in the clinic, and are considered to be the gold standard. The four most frequently systems are the GPS III by Biomet, SmartPReP2 by Harvest, Cascade by MTF, and Magellan by Arteriocyte. These four are the four systems to which ClotMaster was compared. ACP by Arthrex is another commonly used system, but platelet capture rate data was not found for this system. The figure below [Figure 7] provides a comparison of platelet capture efficiency for these four commercial systems and ClotMaster^{TM}.

ClotMaster^{TM} clots, done on gel clots only, were found to have a 92% platelet capture rate. The mean value for the pre-clot samples of whole blood for all 10 volunteers was 187.80 x

$10^3/\mu L$. The mean value for post-clot samples was 4.40 $x10^3/\mu L$. The difference between pre- and post-clot platelet level was compared, and it was calculated that 92% of platelets originally present in pre-clot samples were captured in the fibrin clots.[12]



Comparison of platelet capture rates provides further evidence that the ClotMaster™ clots enhance healing. Platelet capture rates indicate healing potential because those growth factors integral to tissue healing are released from the platelets. Higher platelet capture rates are positively correlated with a higher concentration of growth factors.[8] ClotMaster™ clots capture rate of 92% was more than 20% greater than the leading commercial PRP prep system. This would suggest that ClotMaster™ clots have increased bioactivity compared to PRP and therefore better enhance tissue healing.

DISCUSSION

The positive staining for VEGF, TGF-β1, and PDGF-ββ and the higher platelet capture rate than commercial PRP preparation systems are two strong indications that clots produced by the ClotMaster™ Hula cup have viable tissue healing properties. The fact that these bioactive components are present shows that these clots are capable of enhancing healing at least as well as traditional centrifuge prepared PRP without the expense, long time requirement, and inconsistent results that often limit clinical use.

PRP has demonstrated success as a healing adjuvant in treatment of many wounds including non-healing wounds, surgical wounds, and orthopaedic injuries. Despite demonstrated success over the past two decades, clinical use of PRP is still limited. The limited use of PRP is thought to be due to several major drawbacks to PRP, including the high expense, long preparation time requirement, the need for exogenous activation, and the fact that PRP is a liquid that disseminates following application to the wound site. Also contributing to the limited use of PRP is the inconsistency and incomplete understanding of its contents. As was said earlier, PRP varies drastically depending on preparation method, the patients, and the person preparing it. In addition, the complete makeup of PRP is not well known and understood. It is feasible that clinicians would hesitate to apply PRP to treat a patient without knowing exactly what is in it. Unfortunately, they cannot ever know exactly what is in it because it PRP content changes each time.

Building off the foundation that PRP has built in the clinic, ClotMaster™ clots have the potential to provide equal or better tissue healing enhancement without the major disadvantages of PRP. ClotMaster™ is much less expensive than commercial PRP preparation systems. The clot formation process is extremely simple, takes only 10 minutes, and is repeatable and

consistent. Rather than involving many steps that could go wrong or varyas PRP preparation does, ClotMaster™ can be used by anyone, and involves merely swirling the blood in the cup for a set length of time. Once the contents of the clots are fully analyzed, clinicians will be able to completely understand exactly what they are applying to their patients. Because the clot formation is so simple and consistent, each clot produced will be nearly the same in terms of bioactive components. The only inevitable variant is patients' blood used to form the clot, which also an inevitable variant of PRP. However, the advantage of the adjuvant being completely autologous far outweighs the patient-to-patient variability in clot content. Unlike PRP which requires exogenous activation and therefor contains extraneous contents like xenogeneic bovine thrombin, ClotMaster™ clots are entirely autologous, therefore carrying no risk of immunogenic rejection.

Another advantage of ClotMaster™ clots is that unlike PRP, which is a liquid, ClotMaster™ clots are semi-solid, 3D fibrin based scaffolds. This allows clinicians to directly place the clots into a wound site. Once placed, semi-solid nature of the clots allows for extended physical contact between the clot and the wound, providing prolonged delivery of growth factors. This is not possible with liquid PRP, which can flow freely away from the wound site after injection.

ClotMaster™ clots can also potentially serve as natural, autologous, bioactive scaffolds for tissue engineering. The somewhat porous, fibrin matrix scaffold of the clots and the natural distribution of platelets and growth factors throughout the matrix are excellent features of a biological scaffold. Preliminary cell culture experiments with porcine synoviocytes have shown that clots do function as hospitable scaffolds, as positive cell adherence and proliferation were observed. The function of clots as a biological scaffold is important not only for *in vitro* tissue

engineering, but also for *in vivo* tissue repair. The matrix scaffold structure of the clot provides support for the natural migration and proliferation of cells during the healing process.

One more advantage of the semi-solid robust structure of the ClotMaster™ clots is that surgeons can put sutures through them to anchor them in place, ensuring long-term contact between the wound and clots and therefore ensuring prolonged delivery of growth factors. Orthopaedic surgeon Chris Proctor has demonstrated this use of clots in a number of rotator cuff repair case studies. Due to the structural integrity of the clots, he was able to anchor the clots into the site of the rotator cuff repair.[11]

Lastly, because the Hula Cups and the clot formation protocol can be altered slightly to produce different types of clots, ClotMaster™ provides clinicians with versatility not available in PRP production methods. Further study is needed to determine the key differences between each type of clot including differences in mechanical properties, growth factor content, and platelet capture rates. However, if these differences can be studied and understood, it may be discovered that each type of clot is better for treatment of a different type of wound. By determining the nature of a specific wound and identifying the healing properties required, a clinician could then optimize healing by selecting which type of clot to produce and apply.

Figure 11. Summary table of advantages of ClotMaster™ clots[11]		
Biologic Advantages	**Technical Advantages**	**Cost Advantages**
• Rich in platelets • Structural integrity of fibrin matrix • Rich in growth factors (TGF-B1, PDGF-BB, VEGF, FGF2) • Possible to use as scaffold for TE • Completely autologous (no exogenous activation required)	• Quick • Easy procedure • Consistent results • Requires no extra OR staff • Can suture to repair site • Versatility (different types of clot)	• Inexpensive

FUTURE DIRECTIONS

The next step towards characterizing the tissue healing properties of ClotMaster™ clots will be to determine whether the TGF-β1 found is in its latent or active form, and whether or not platelets are activated during clot formation. Another downside to traditional PRP is that the preparation process involves exogenous activation of platelets, causing a rapid burst of platelet releasate and activation of TGF-β1.[5] This leads to a very brief exposure period, as the plasma half-life of TGF-β1 is approximately 2 minutes and all platelet-derived growth factors are released within an hour of activation.[13] If the ClotMaster™ clots contain LAP-TGFβ, because there is not exogenous activation prior to application, it will be activated *in situ* causing a more sustained release, and therefore longer dosing times to improve healing. If platelets in the clot are not activated, they too will be activated *in situ* leading to a prolonged release of growth factors.

In addition to testing whether or not TGF-β1 and platelets are activated during clot formation, to better understand the contents of the clots, all growth factors must be quantified using ELISA. While TGF-β1, PDGF-ββ, VEGF, and FGF2 are the major four growth factors in PRP and in healing, other growth factors also likely exist. Immunohistochemistry to detect EGF, IGF, PDGF-αβ, and other growth factors that play a role in healing should be run. Once these growth factors are shown to be present, ELISA should be conducted on all present growth factors to get an idea of the concentration of each growth factor in the clot.

In order to most directly and accurately compare ClotMaster™ clots to PRP, a study could be done using the same set of patients, the same immunohistochemistry and ELISA tests, and the same method of measuring platelet capture efficiency. Blood could be obtained from each of a set of patients, half for PRP production using the gold standard commercial preparation system and half for ClotMaster™ production. Both clots and PRP would be produced from each

patient's blood, and then PRP and clot products would be analyzed using the same techniques for growth factor presence and concentration and for platelet capture rate. Results for the clot product for each patient would be compared to the PRP product of each patient. This type of experiment would control for many of the confounding variables previously preventing comprehensive comparison including the patient, experimenter, PRP prep system, IHC and ELISA technique, and platelet capture rate measuring method. By conducting such a controlled experiment, differences between PRP and ClotMaster™ clot would be clearly shown and understood.

Furthermore, differences between different types of clots should be investigated more deeply. It is clear that the dense clot and gel clot have very different physical properties and somewhat different growth factor contents. Further studies examining the mechanical strength and properties of each of the clots and further looking at concentration of growth factors and platelet capture rate would be useful. If notable differences in the bioactive and physical properties of each clot are found, it may be found that each clot is best for treating certain types of wounds. Once the differences between clots are understood, clinicians could select which clot to produce and thereby further optimize healing.

Although ClotMaster™ clots are very new in the field compared to PRP, the potential they have in advancing healing is undeniable. Further investigation of their properties is highly warranted. By studying the bioactive components of clots and comparing them to PRP, not only can scientists and clinicians learn more about the clots but they can also learn much more about the long mystifying content of PRP. Learning more about PRP, ClotMaster™ clots, and autologous blood derived healing adjuvants in general is a necessary step in the advancement of wound treatment.

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