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Physiology of Blood Components in Wound Healing: an Appreciation of Cellular Co-Operativity in Platelet Rich Plasma Action

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Abstract

Pain management and injury repair represent areas of significant clinical unmet need in Orthopedics and Sports medicine, especially in the management of chronic and degenerative connective tissue diseases such as osteoarthritis and tendinopathies. Trends in the clinical data from recent studies show autologous platelet-rich plasma (PRP) therapy is emerging as a safe and effective means for improving patient outcomes. However, the precise mechanisms through which PRP exerts its effects remain elusive, and there exists much controversy in the literature over what might comprise an "optimal" PRP formulation. Part of the controversy surrounding PRP formulation is there are many commercially available devices for PRP preparation that generate vastly different products that are not equivalent in cellular or biochemical composition. The range of different PRP products has led to the need for classification systems to indicate whether a given PRP contains high or low platelet concentrations, what the relative leukocyte content is, and whether it contains red blood cells (RBCs) as examples. Although autologous PRP has proven safe and effective in improving patient outcomes in a broad range of clinical applications regardless of formulation, a current area of active debate relates to the role of non-platelet cells in the mechanism of action of PRP, and in particular to what extent leukocytes and RBCs may enhance or detract from the presumed benefits of platelet action. Although very little is actually known about the roles of non-platelet blood components in the activity of platelets with respect to PRP therapy, there is a substantial body of literature relating to the roles of blood components in the physiology of normal wound repair. In a normal wound repair reaction all of the components of blood have important roles in driving the healing cascade, and the co-operative physiological balance of cellular and biochemical action results in restoration of the injured tissue to a healthy state. This narrative review article discusses the salient roles of each of the major blood components in normal wound repair and how these foundational principles may also relate to the mechanisms of PRP action.

Key Words: Platelet Rich Plasma, Wound healing cascade, Growth factor, Leukocyte, Osteoarthritis, Tendinopathy, Physiology, Hemostasis, Inflammation, Proliferation, Remodeling, Regenerative medicine.

Introduction

To better understand how Platelet Rich Plasma (PRP) can impact chronic pain and initiate wound repair, it is important to gain an appreciation for the normal biology of wound healing and how each component of whole blood, from which PRP is derived, can contribute to a physiologic tissue response to drive the healing cascade. This review provides information on the salient roles of blood components as they relate to pain management and injury repair.

PRP is a multi-cellular platelet concentrate derived from autologous whole blood, with platelet concentrations elevated above baseline whole blood levels[1,2]. Platelets within PRP contain a high concentration of bioactive factors that are stored inside of granules that can be released to activate acute inflammation and stimulate the healing cascade[3-7]. Depending upon how PRP is generated it can contain additional blood cell types such as white blood cells (WBCs), red blood cells (RBCs), and circulating stem cells, which can all contribute to the bioactivity and healing potential of the PRP[8,9]. Plasma is the straw colored fluid component of whole blood that suspends the cellular components and contains clotting factors and essential nutrients.

KEY CONCEPT: Platelets are not the only active component in PRP. All components of whole blood have important roles in the physiology of wound healing

Components of Whole Blood

Human blood is comprised of plasma and three main types of cells; RBCs, WBCs, and platelets (Figure 1). The dynamic interactions between these cells are crucial for maintaining normal health, providing the physiological mechanisms to ensure survival and promote healing. [6,10-25]

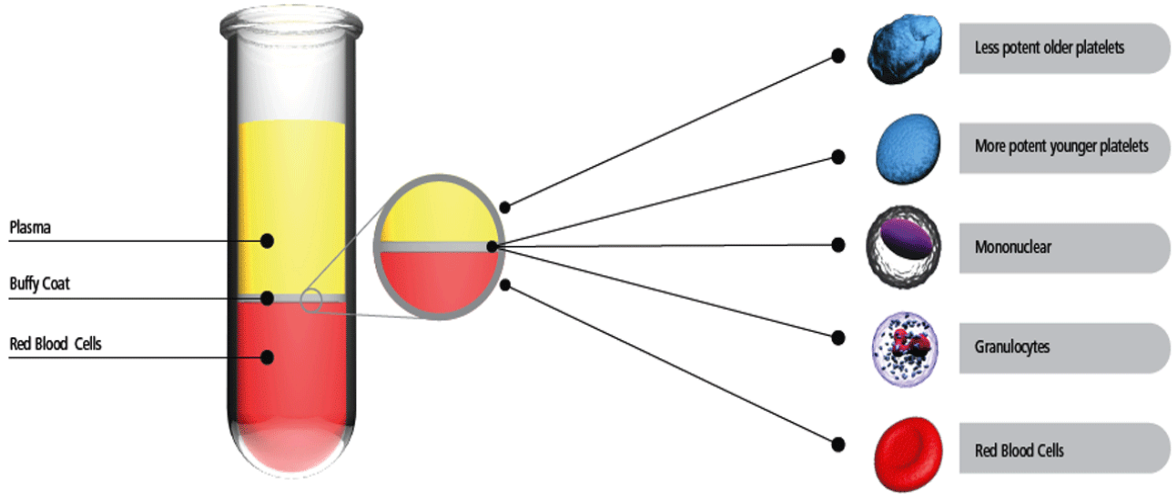

Figure1. Schematic representation of the components of centrifuged whole blood Whole blood. separates into 3 distinct layers when sufficiently centrifuged. In order of increasing density, these layers constitute plasma at the top of the density gradient followed by a thin whitish "buffy coat" layer, and compacted red cells at the bottom. Blood cells are further divided based on their position with respect to the "buffy coat". Platelets have a broad range of density depending primarily on their age, with less dense older platelets that contain fewer granules and thus fewer growth factors and cytokines in the plasma and in the uppermost fraction of the buffy coat. Less dense platelets are preferentially collected by leukocyte-poor PRP systems that avoid collecting cells from the buffy coat. Younger and more potent platelets significantly overlap in density with mononuclear leukocytes. The Mononuclear leukocyte fraction includes lymphoctyes, monocytes, and peripheral blood stem cells. Mononuclear leukocytes are long-lived cells and can reside in tissues for many weeks following injury and are thought to provide a source of extended-release growth factors during a wound healing reaction. Monocytes, which are progenitor cells that differentiate into macrophages and dendritic cells, are particularly important for the transition from the inflammatory phase of healing to the proliferation phase. Peripheral blood stem cells may have important roles in providing a source of progenitor cells that can aid in guiding the healing response toward repairing tissue damage. Granulocytes, including neutrophils, eosinophils, and basophils are the most dense of the buffycoat cells. In fact granulocytes can have significant overlap with the density of red blood cells depending upon their absolute granule content. Granulocytes, and the very abundant neutrophils in particular, are integral to the innate immune system. These cells have the primary responsibility of host defense against invading pathogens and foreign bodies.

Plasma: Plasma is the fluid component of blood that has three primary functions.

1.Transport of nutrients, metabolic waste products and endocrine factors.

2.Transport of blood cells.

3.Provide clotting factors such as fibrinogen and pro-thrombin to support coagulation

Platelets: Platelets are non-nucleated cells derived from very large precursor cells called megakaryocytes that reside in the bone marrow. During their development, platelets obtain large numbers of storage granules that contain different growth factors, cytokines, and hormones required for wound healing. [4,5,7,26,27] Platelet activation is a highly regulated process that culminates in degranulation, or the release of granule contents.[5,28-32] The process of degranulation is a key step in wound healing because the growth factors and other mediators that platelets release program damaged tissue for repair.[29-34] In a normal healthy state, platelets and WBCs circulate in inactive forms. However, in pathological states, such as an injury involving blood vessels, platelets become activated by contact with components of the extravascular connective tissues that are exposed at the site of injury.[35,36] Collagen and Tissue Factor are examples of tissue components that activate platelets. [37]

KEY CONCEPT: Platelets and leukocytes have coordinated and cooperative activities in normal wound healing that limit acute inflammation and trigger tissue repair.

Under injury conditions, platelets have two essential functions for survival.

1.Hemostasis: Drive clotting to stop the bleeding. [17,38]

2.Inflammation: Initiate healing by releasing growth factors and bioactive molecules that activate acute inflammation and program tissue repair. [11,17,39-41]

WBCs and platelets become activated together in a physiological context for wound repair. [6,17,42] This coordinate activation facilitates crosstalk that permits the modulation and control of each other's activities. This results in a balanced and biologically optimized tissue repair response to injury. [6,17,39,42]

WBCs: The primary role of WBCs is to decontaminate the wound and prevent infection. WBCs also debride the wound of dead and damaged tissue, and ultimately deposit and activate growth factors that direct the conversion of a fibrin clot into vascularized and viable tissue. [30,40,41,43-47] There are two main classes of WBCs.

1.Granulocytes: Include neutrophils that engulph foreign bodies, produce immune regulating cytokines, lipids, and proteases, and

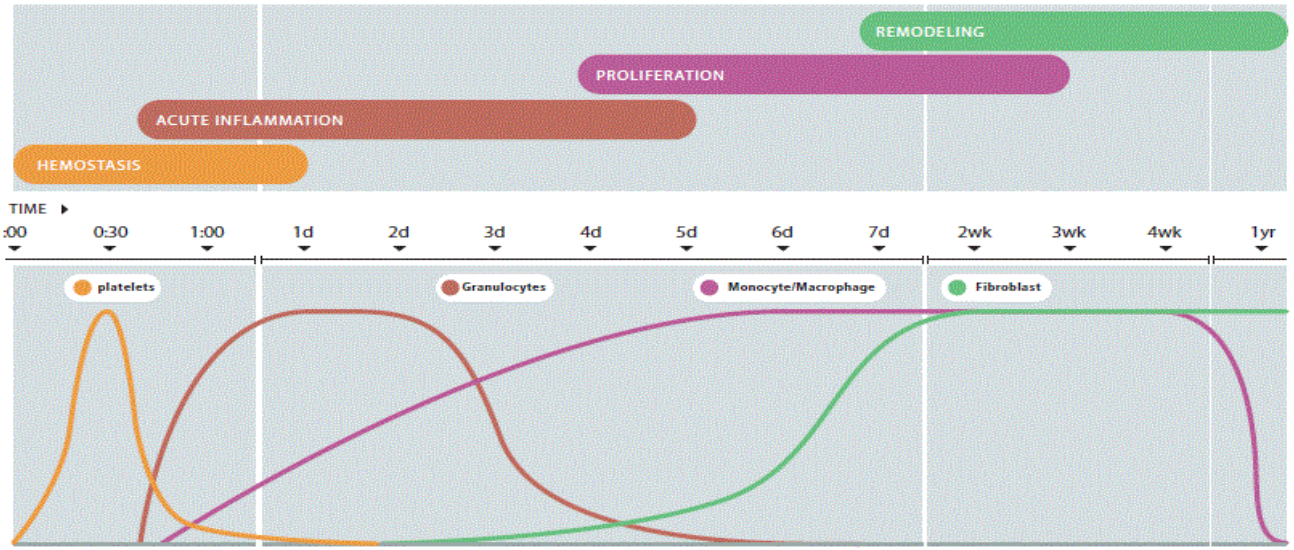

Figure2: "The wound healing cascade consists of 4 partially overlapping phases; hemostasis, acute inflammation, proliferation, and remodeling. The activity of a particular cell type within each phase is crucial for the progression and successful execution of the healing cascade, ultimately leading to tissue repair. Hemostasis, the shortest phase of the healing cascade, is the primary responsibility of platelets. Platelets are integral to hemostasis but also function as the physiological trigger to activate acute inflammation. The acute inflammation phase of wound healing involves the mobilization of innate immune cells to decontaminate and debride the wound. The activity of granulocytes is tightly regulated, and these cells require multiple activation steps in order to drive an inflammatory reaction. In the absence of an activating signal, primed neutrophils for example actually play an important role in quenching inflammation and actively accelerate transition into the proliferation phase of the healing cascade by promoting monocyte differentiation into anti-inflammatory macrophages that are specialized for phagocytosis and guiding tissue repair. The proliferation phase is initiated by macrophage activity that recruits fibroblasts, endothelial cells and stem cells into the forming granulation tissue. This is primarily driven by macrophage sustained release of growth factors such as TGF- β, EGF and VEGF. During the proliferation phase, macrophage driven fibroblast activity replaces the fibrin matrix with a more durable type 3 collagen matrix. The type 3 collagen matrix facilitates the budding and growth of new blood vessels that is driven by macrophage factors such as VEGF. The proliferation phase culminates in the establishment of a vital and vascular granulation tissue. The remodeling phase is the longest phase of the healing cascade and may last for several months or years depending on the nature and severity of the initial injury. Fibroblasts are responsible for remodeling, and this consists of replacing the type 3 collagen matrix with the stronger type 1 collagen matrix. During the process of remodeling, type 1 collagen fibers become aligned to the direction of force within the tissue by specialized fibroblasts called myofibroblasts. Myofibroblasts are contractile cells, and as such can sense and resist forces exerted on the tissue. Ultimately, collagen realignment restores strength and function to the repair tissue, which evolves into a mature and relatively avascular scar."

release antimicrobial granules. [44,48-51]

2.Mononuclear cells: Include monocytes and lymphocytes that release factors to control inflammation and regulate cell growth for days to months following injury. [12,49,50]

RBCs: The primary role of RBCs is to carry oxygen to tissues to support metabolism, and carry carbon dioxide waste away to prevent acidification. In wound healing, RBCs act in an amplification loop to increase the activation and release of bioactive factors from platelets. [15,22,23,52]

Peripheral Blood Stem Cells: Multipotent mesenchymal stem cells (MSCs) are capable of differentiating into multiple cell types. MSCs also secrete factors that control inflammation and promote tissue repair. [53-59] These MSCs co-localize with leukocytes based on their density into the buffycoat fraction of centrifuged blood.[60] Therefore, leukocyte poor PRP products are also lacking in peripheral blood stem cells.

Wound Healing Fundamentals

The wound healing process begins when normal healthy tissue is injured. Tissue can become injured through different mechanisms, for example a cut, puncture, blunt trauma, or even excessive use or overloading. When an injury occurs in vascular tissue, blood may leak from damaged vessels into the tissue. It is this bleeding that triggers the healing cascade.[30,40,45-47] Under normal circumstances, the healing cascade is a well-orchestrated and highly regulated series of four interdependent steps (Figure 2).[30,40,45-47] These four steps lead to the repair of damaged tissue and its restoration to a normal healthy and functional state. The four phases of the wound healing cascade occur over weeks to months depending on the magnitude and severity of the injury. These phases include:

1.Hemostasis- clot formation.

2.Acute Inflammation- Platelet activation and immune mobilization

3.Proliferation- Cell multiplication and matrix deposition.

4.Remodeling- Scar formation and tissue restoration.

Each phase is dominated by a particular cell type that prepares the tissue for the events in the next phase (Figure 3). [30,40,45-47] It is important that each phase is executed effectively to ensure transitions between phases occur properly. If this does not happen, the repair process may be subverted into chronic and

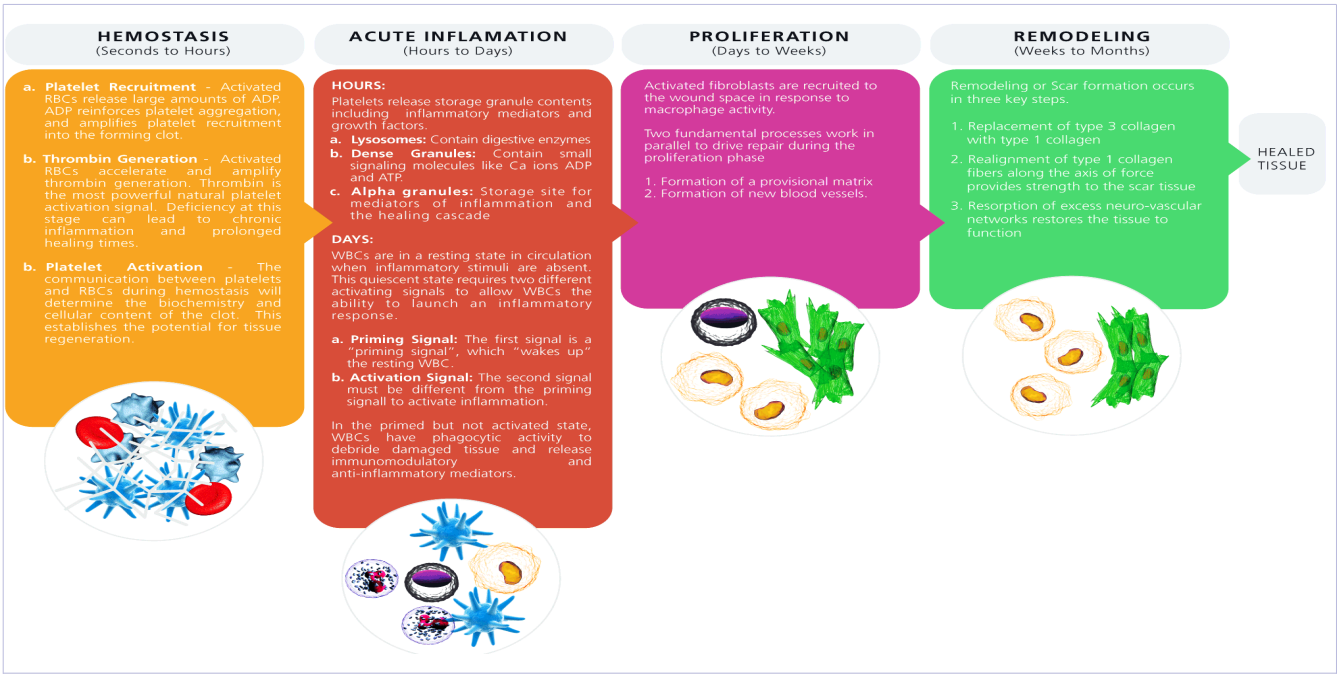

Figure 3. Cellular contributions to the healing cascade.

potentially degenerative pathological states.[39,41,46,49,61-63]

Hemostasis

The first and shortest phase of the wound healing cascade is hemostasis.[46,64] Hemostasis is the process of forming a blood clot to stop bleeding. All of the factors required for blood clotting reside in the plasma.[65] These factors include calcium ions, enzymes of the clotting cascade, fibrinogen, and pro-thrombin. The overarching gate-keeper to clot formation isthe generation of thrombin from its inactive form pro-thrombin. [64,65] Once activated, thrombin can catalyze the conversion of fibrinogen to fibrin. Fibrin then polymerizes into branched fish net-like fiber arrays that are able to capture and concentrate platelets and other blood cells.[66-69] .

KEY CONCEPT: Thrombin represents a critical hinge between hemostasis and acute inflammation. RBCs play an important role in amplifying thrombin generation to ensure efficient and effective execution of both phases of the wound healing cascade.

Under physiological conditions, the primary signal to generate thrombin is provided by platelets when they encounter collagen and substances that are not normally found inside the blood vessel (Figure 4).

[38,70] Partial activation of platelets by collagen causes a shape change that makes platelets stick together (aggregation) and adhere to the site of injury.[37,46,70,71] Aggregation stimulates platelets to begin releasing factors that reinforce their aggregation and adhesion, and promote further recruitment of platelets into the forming clot known as a "platelet plug" or thrombus.[35,72] Among the platelet factors released during the initial hemostasis response, ADP (adenosine di-phosphate) and LPA (lyso-phosphatidic acid) are particularly important because they can activate RBCs. [22,23,52,73,74].

Activated RBCs influence three important actions that contribute critically to the healing cascade.

Platelet Recruitment

Activated RBCs release large amounts of ADP. [22,23,52,73] ADP reinforces platelet aggregation, and amplifies platelet recruitment into the forming clot.[22,23,52,73] This activity may ensure the formation of a robust platelet plug that can stop the bleeding and contract the wound. The resulting increase in platelet concentration also provides the injured site with a large pool of growth factors and inflammatory mediators.

Thrombin generation

Activated RBCs accelerate and amplify thrombin generation. This RBC-driven activity stems from the exposure of phosphatidyl serine (PS) on the surface of the activated RBC. [23,52,70,73] PS is a negatively charged lipid that is normally stored inside of the RBC. Its exposure on the cell surface directly activates thrombin generation.[52] This is a particularly powerful feed-back amplification mechanism because RBCs have a very large surface area compared to platelets. High thrombin concentrations are required to accelerate fibrin polymerization into a stable clot. [67,69]

Platelet Activation

Thrombin is also the most powerful natural platelet activation signal, so without robust thrombin generation, platelet activation and growth factor release is deficient.[75-78] Because platelet activation provides the initial stimulus for the healing cascade, deficiency at this initial stage can lead to chronic inflammation and prolonged healing times. [62,79]

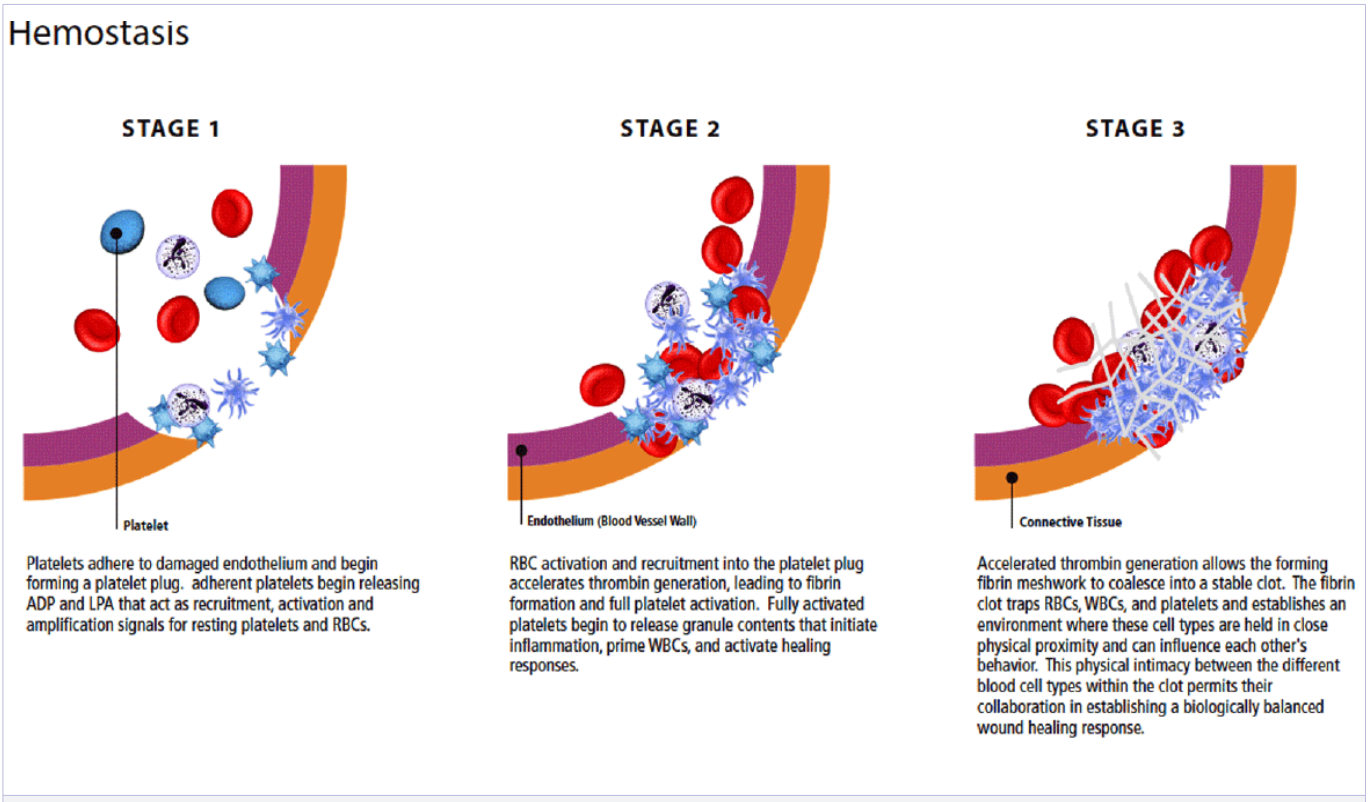

Figure 4.Stages of hemostasis. Platelet interactions with RBCs and WBCs determine the cellular and biochemical properties of the forming blood clot. These clot properties established during hemostasis will impact the efficiency of the healing cascade and the quality of the resulting repair tissue.

The communication between platelets and RBCs to activate robust thrombin generation during the hemostasis phase therefore determines much of the biochemistry and cellular content of the clot.[15,52,67] This cooperative activity establishes the potential for the injured tissue to heal, and sets the stage for the next phase of the healing cascade.

Acute Inflammation

Acute inflammation in response to injury is a platelet dependent reaction triggered simultaneously with the activation of hemostasis.[4,11,18,24,41,46,72,80] However, unlike hemostasis that comes to completion within minutes, acute inflammation typically lasts from four to fourteen days.[40,45,46,62] The duration and magnitude of the acute inflammation phase is thought to be dependent on the extent of injury and whether the wound has been significantly contaminated with microbes or foreign bodies.[62] The physiology of WBCs is markedly different in the presence of microbial or foreign body contamination than in sterile inflammation or at rest. This section will focus on the biology of sterile acute inflammation such that occurs in a subcutaneous bruise or in an uncomplicated surgical wound because this state of sterile acute inflammation is also relevant to a PRP injection that has been conducted properly under aseptic conditions, without introduction of microbial contamination into the treated tissue.

The evolutionary pairing of hemostasis and acute inflammation occurs through the action of thrombin. Thrombin helps to ensure the acute inflammatory response is proportional to the magnitude of the injury.[81,82] Maintaining balanced wound healing physiology is an important concept as insufficient inflammation may result in poor or delayed healing, while excessive inflammation may interfere with healing by causing additional tissue damage. [45,46,62]

The acute inflammatory phase of the healing cascade begins in earnest when platelets become fully activated by thrombin and degranulate, releasing inflammatory mediators and growth factors from platelet storage granules.[4,5,30,83,84]

Platelets contain three types of storage granules. [5,83]

a.Lysosomes: Contain digestive enzymes

b.Dense Granules: Contain small signaling molecules like Calcium ions, ADP and ATP.

c.Alpha granules: Contain hemostatic factors, growth factors and inflammatory mediators.

Alpha granules are the largest and most prevalent granule type in the platelet. These granules serve as storage vessels for effectors of the healing cascade. Platelet growth factors released from alpha granules are largely responsible for the role that platelets play in directing injury repair and wound healing (Table 1).[4,5,7,24,30,72,83,84] Platelets release their alpha granule contents with an initial burst, and exhaust most of their stored cargoes within the first hour after activation.

Table 1. Known functions of key platelet growth factors.

platelet growth factor	known function
PDGF-AB	Stimulates cell proliferation Promotes angiogenesis Promotes epithilization Potent fibroblast and immune cell recruitment factor
TGF-β1	Promotes extracellular matrix synthesis Potent immune suppressor
VEGF	Stimulates endothelial cell proliferation Promotes angiogenesis
EGF	Stimulates angiogenesis Promotes epithilization Promotes cell differentiation and maturation Promotes collagenase activity and tissue remodeling
SDF-1α / CXCL12	Potent stem cell recruitment factor Monocyte recruitment factor Neural cell progenitor recruitment factor Augments angiogenic effect of VEGF

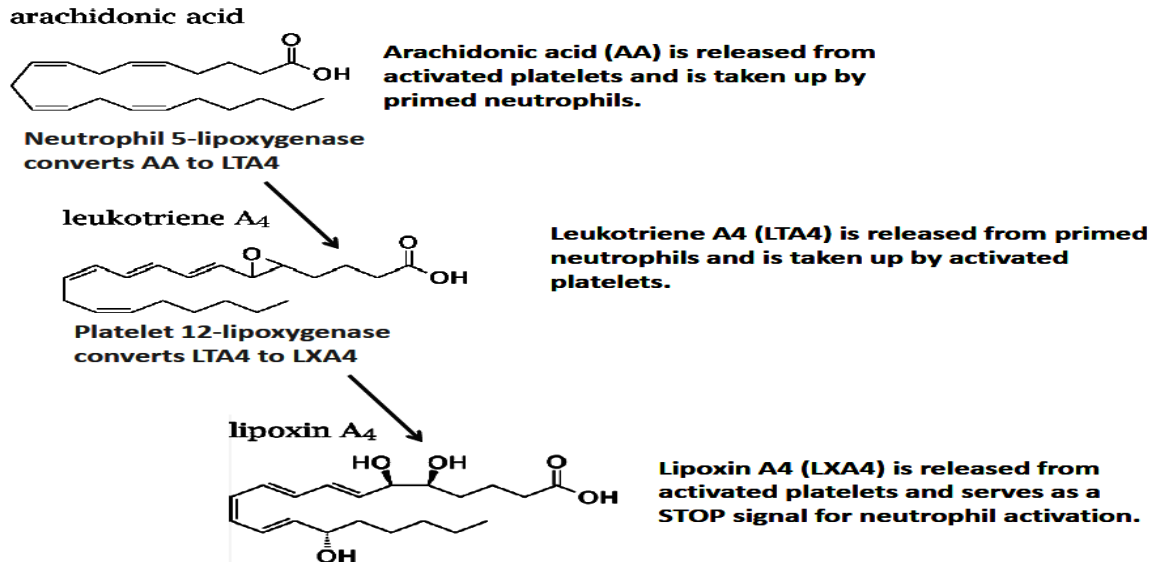

Figure 5. Platelet-neutrophil transcellular biosynthesis of anti-inflammatory and pro-resolving lipid mediators. Platelets and leukocytes collaborate in the synthesis of anti-inflammatory and pro-resolving lipid mediators in order to control the magnitude and duration of the acute inflammatory phase. Platelet activation triggers the production and release of arachidonic acid (AA), which is taken up by neutrophils as a starting material for production of prostaglandins and leukotrienes. These inflammatory lipid mediators induce pain and swelling, and exacerbate inflammation by increasing the infiltration and activation of neutrophils from the circulation. In particular, neutrophils can convert platelet-derived AA to leukotriene A-4 (LTA4). LTA4 is then taken up by activated platelets where it is further converted to lipoxin A4 (LXA4).LXA4 is a potent inhibitor of neutrophil inflammatory activation, and serves as a powerful brake to prevent further neutrophil infiltration. This cooperative lipid biosynthesis pathway forms a key regulatory element of the transition from the acute inflammatory phase to the proliferative phase of the wound healing cascade. Through such cooperative mechanisms, platelets and neutrophils can work together to limit the magnitude and duration of the acute inflammatory response and prevent collateral tissue damage that may occur from excessive inflammation in wounds that are free of contamination and foreign bodies.

[4,5,30,72,83,84] Platelets have a limited capacity to synthesize new proteins.[85-87] Therefore, their major contribution to the healing cascade is to activate the acute inflammatory response to program damaged tissue for debridement and repair. The limited growth factors platelets do continue to produce after degranulating is believed to facilitate the control of WBC activities inside the clot.[86,87] This mechanism can guide WBC behavior toward debridement of dead and damaged tissue while preventing excess inflammation especially from neutrophils, when no microbial contamination is present.[88]

KEY CONCEPT: Activated platelets and primed neutrophils cooperate in the biosynthesis of factors that control the magnitude and duration of inflammation.

An additional cooperative lipid-mediated anti-inflammatory pathway between activated platelets and platelet primed neutrophils has also been identified (Figure 5).[79,89,90] Thistrans-cellular metabolic pathway involves the generation of arachidonic acid derived lipid mediators that modulate inflammation and promote wound healing.[79,89,90] More specifically, the attachment of activated platelets to neutrophils allows neutrophils to take up arachidonic acid that is released by activated platelets. Neutrophils can convert this platelet-derived lipid to various prostaglandins (PGs) and leukotrienes (LTs).[91] PGs can contribute to pain and tissue swelling, while LTs typically act as potent chemotactic signals for the recruitment of immune cells.[92,93] However, platelets attached to neutrophils that generate LTs can quickly take up these inflammatory mediator lipids and convert them to lipoxins (LXs).[91] Lipoxins are very potent anti-inflammatory molecules that play important roles in limiting neutrophil activation, preventing their migration from vessels into tissues, and in driving the resolution of inflammation. [79,89,90,94] It is important to note that platelets lack the ability to synthesize lipoxins without the LTs intermediates produced by neutrophils.[91] In addition, neutrophils can be induced by PGs to switch the arachidonic acid lipid metabolites they generate from pro-inflammatory LTs directly to LXs.[95,96] This switch from generation of pro-inflammatory lipid mediators to anti-inflammatory lipid mediators in neutrophils is thought to prevent further neutrophil recruitment and inflammatory activation while simultaneously activating resolution pathways that can accelerate healing. [79,90,91,96] Transcellular cooperative regulation of the magnitude and duration of the inflammatory response may begin to explain why platelet-leukocyte ratios are emerging as important parameters in the therapeutic efficacy of platelet-rich plasma products.[34,44,66,76,97,98]

WBCs

WBCs are in a resting state in circulation when inflammatory stimuli are absent. This quiescent state requires two different activating signals to allow WBCs the ability to launch an inflammatory response (Figure 6).[99-105]

Priming Signal

The first signal is a "priming signal", which "wakes up" the resting WBC. In the context of a sterile injury, this priming signal can be provided by activated platelets.[100] Following the priming signal, WBCs become competent to communicate with their environment and gain the ability to generate and release an array of cytokines and growth factors that can modulate the activity of other cells.[10,11,51,88,99,100,106-108] This is important because after platelet growth factor release is exhausted early in the acute inflammation phase it is predominantly the activity of WBCs that guide the healing cascade forward to proliferation and remodeling.[39,40,44-47,62,83] Interestingly, new evidence shows that the leukocyte priming reaction is actually reversible. [102,104,109,110] This may allow WBCs greater flexibility in how they respond to signals in the environment to drive wound healing, as priming does not always lead to activation and a robust inflammatory response. This phenomenon may play a key role in the self-limiting nature of platelet-driven acute inflammatory reactions, and could be one pillar to help explain the observation that leukocyte-rich PRP does not exacerbate inflammatory cytokine levels in osteoarthritic joints.[111]

KEY CONCEPT: Neutrophils require separate priming and activation signals to elicit an inflammatory response. In the context of an aseptic tissue injection there is no separate neutrophil activation signal available.

Activating Signal

In the context of a sterile injury such as a bruise or a platelet-rich plasma injection, there is no activating signal present to drive WBCs to an inflammatory state. This is due to a fail-safe evolutionary adaptation to control against excessive tissue damage that is built into WBC physiology. The activating signal must either be different from the priming signal or be of significantly greater magnitude than the original priming signal. [100]

In the case of leukocyte-rich PRP without prematurely activated platelets, WBCs have been harvested from the circulation where they are in a resting state. Therefore the WBCs in the PRP remain in a resting state when they are introduced to the tissue. In this case, WBC priming occurs upon collagen-mediated platelet activation within the treated tissue.[11,17,83] Therefore, all aspects of the pathological state within the tissue being treated are simultaneously part of the leukocyte priming reaction. Thus, no separate activating signal is present to drive the injected WBCs to an excessive inflammatory state. This theory sheds light on why autologous platelet rich plasma preparations do not present a significant safety risk when used therapeutically, regardless of their leukocyte content.[1,31-34,44,112-116]

In the primed but not activated state, WBCs have enhanced phagocytic activity that promotes debridement of damaged tissue and the release of a large array of immunomodulatory and anti-inflammatory mediators such as TGF-b1 and IL-1RA as well as lipid modulators of inflammation like lipoxins and resolvins.[79,90,117]

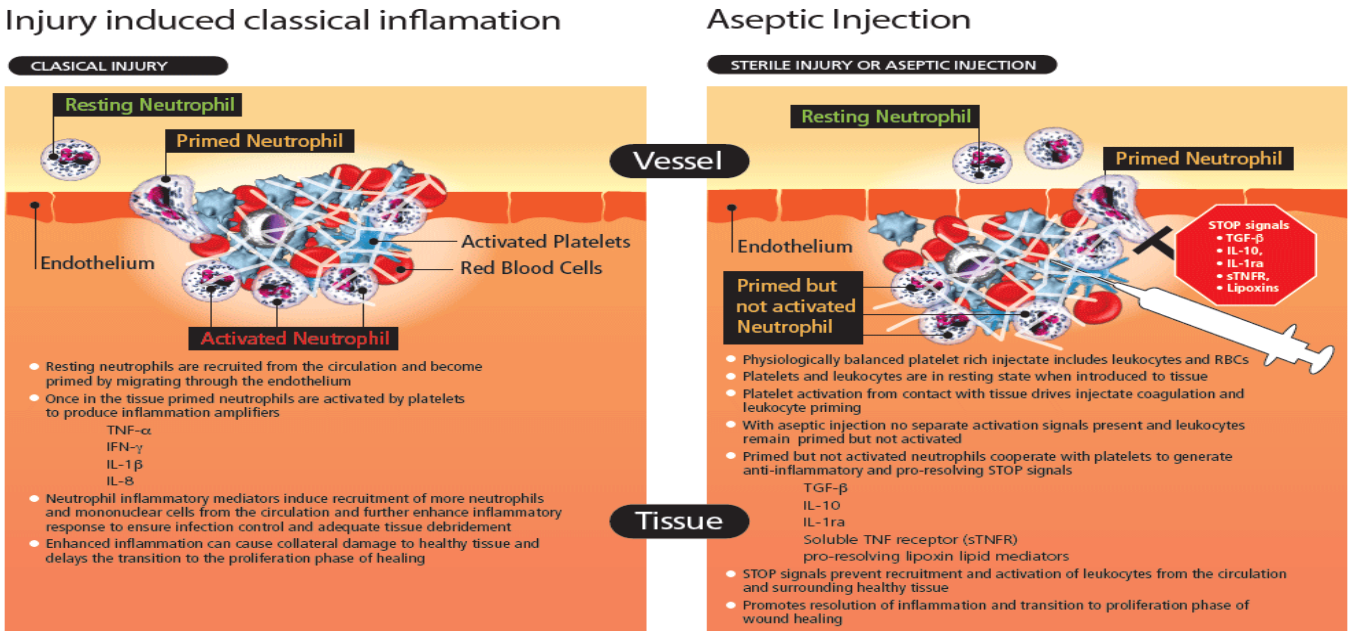

Figure 6. Representation of key differences in acute inflammation between classical injury and sterile injury or aseptic Injection Neutrophils require at least 2 sequential signals to prime them for activation and subsequently trigger an inflammatory response. In a classical injury-induced inflammatory response, neutrophils are recruited to the site of injury by chemotactic or noxious stimuli in a process that involves migration across the endothelium into the damaged tissue. In this scenario, neutrophil priming occurs during migration into the damaged tissue. Because neutrophils enter the tisssue in a primed state, inflammatory mediators and platelet products can cause neutrophil activation leading to inflammatory cytokine responses and even the oxidative burst reaction. In this scenario, neutrophils can cause collateral damage to healthy tissue, exacerbating the injury and potentially delaying transition to the proliferation phase of the healing cascade.
In contrast, neutrophils contained in a PRPinjection, for example, are in a resting state when introduced to the tissue and still require priming before they can be activated to launch an inflammatory response. Priming can be accomplished when platelet activation and degranulation occurs within the injected tissue. In this scenario, platelet-driven neutrophil priming would occur in the context of whatever inflammatory mediators are also present in the injected tissue. Therefore, no additional neutrophil activating signals are present and so the injected cells remain in the primed, but not activated, state. In this state, neutrophils have an enhanced phagocytic activity and are competent to cooperate with platelets in controlling the magnitude and duration of inflammation by producing STOP signals that prevent further immune cell recruitment from the circulation and the surrounding tissues. This activity promotes resolution of the inflammatory phase and accelerates the transition to the proliferation phase of wound healing.

These mediators can actively suppress chronic inflammation and prevent the migration and recruitment of new leukocytes into the treated tissue, thereby directing cellular activities toward tissue repair.[79,90,117-121] In contrast to resting leukocytes that may be injected with LR-PRP, leukocytes that have been recruited from the circulation through the vessel wall and into the tissue in response to inflammation are primed by this migration and may become activated once in the tissue and further exacerbate inflammation.[122] In this context, activated WBCs could potentially drive excessive inflammatory reactions that can lead to fibrosis and delayed healing.[46,49,62,63] From this perspective, it is increasingly important that mediators released from primed but not activated leukocytes can suppress inflammation and prevent the further recruitment of activated cells.[79,90,117-121]

The requirement for separate WBC priming and activation signals and the anti-inflammatory activities of primed but not activated neutrophils can in part explain the clinical literature regarding the emerging greater efficacy of leukocyte rich PRP treatment compared with leukocyte poor PRP to relieve pain and improve function in osteoarthritis and in tendinopathy patients.[113,114] Indeed, patients with an array of soft tissue pathologies have been shown to benefit from LR-PRP injections without any significant associated adverse events despite containing concentrated leukocytes.[113-115]

Proliferation

The proliferation phase begins two to three days after injury and is marked by the activation of fibroblasts by inflammatory mediators and growth factors released during the acute inflammatory phase.[29,39,40,46,62] The process of fibroblast migration from the wound margins into the fibrin clot matrix is initiated by growth factors such as PDGF,TGF-b1, and FGF that are generated by monocyte-derived macrophages. [39,44-46] At this point in the healing cascade, the ability of these leukocytes to generate new growth factors over time becomes crucial. This is because the pool of platelet growth factors that were released during the inflammatory phase is thought to be short-lived and becomes depleted.[40,83]

KEY CONCEPT: Protease activity does not simply destroy extracellular matrix and cause degeneration. Protease cleavage inactivates pain mediators and inflammatory cytokines, and activates growth factors for proliferation, angiogenesis, and new matrix synthesis.

Protease activity from macrophages and fibroblasts also becomes critical at this point in the healing cascade as the fibrin matrix must be partially digested to allow the migration of cells in the wound.[45,46,123] Additionally, key growth factors such as IGF-1, TGF-b1 and VEGF depend on extracellular protease activity for their bioavailability.[123-125] Moreover, protease cleavage also targets the destruction of key inflammatory mediators like IL-1b and MCP-3 (CCL7), and as such functions to dampen inflammation and promote transition to the proliferation phase. [50,118,125] Therefore, non-selective strategies such as α2-macroglobulin therapy that aim to inhibit protease activity to improve the strength of the matrix may in advertently interfere with tissue repair and delay healing. [123,125] Interestingly, of the matrix metalloproteinases, MMP-2 (neutrophil gelatinase A) activity in particular appears to play key functional roles in promoting the growth of new blood vessels and in driving wound resolution.[123] This may be in part due to the ability of MMP-2 to inactivate inflammatory mediators like IL-1b and MCP-3 and to release critical growth factors like TGF-b1 and IGF-1 from their tissue inhibitors.[48,106,123-127] The catalytic activity of MMP-2 also unlocks the anti-inflammatory potential of TGF-b1. [48,106,124,126] In addition, MMP-2 can also inactivate powerful neuro-inflammatory mediators of chronic pain such as Calcitonin Gene-Related Peptide (CGRP).[128] CGRP is thought to play an important role in the chronic pain of osteoarthritis by causing vasodilation that contributes to effusion, and by causing persistent sub-acute synovitis and hyperalgesia.[129,128] It is therefore noteworthy that recent data indicate that levels of active MMP-2 are greater in leukocyte-rich PRP releasates compared with leukocyte poor.[130] This finding should not be considered surprising since neutrophils represent the richest source of MMP-2, also known as neutrophil gelatinase A.

Granulation tissue consists primarily of fibroblasts and new blood vessels.[39,40,45-47] Two fundamental processes, provisional matrix deposition and the formation of new blood vessels (angiogenesis), work in parallel to drive repair during the proliferation phase. Angiogenesis lags slightly behind new matrix formation.[40,45-47,131] Oxygen is critical for fibroblasts to produce collagen in order to establish granulation tissue.[132] Thus, re-vascularization is a key rate-limiting step in the healing cascade. A wide range of growth factors and chemical mediators have been identified that influence the developing capillaries. These include macrophage derived factors like TGF-b1, PDGF, VEGF and FGF.[39,40,44-47,131,132]

In the wound, fibroblasts initially produce type III collagen, which provides a weaker and less extensively cross linked tissue matrix than the type I collagen of the uninjured or mature repair tissue.[131] Collagen III stabilizes the forming granulation tissue, but it is easier for fibroblasts, endothelial and immune cells to migrate through.[131] This facilitates repopulation of the granulation tissue with viable cells. Type III collagen will be replaced in the matrix with type I collagen as healing progresses from proliferation to remodeling. [39,40,46,47,62,131]

Towards the end of the proliferation phase, fibroblasts are driven by macrophage signals, predominantly TGF-b1, to transdifferentiate into myofibroblasts.[49,133-135] Myofibroblasts are specialized cells that generate new matrix but also become contractile through the expression of smooth muscle actin. Contraction is important because it provides mechanical strength to the granulation tissue and reduces the wound size.[133-135] An interesting property of myofibroblasts is that contraction triggers their generation of TGF-b1. [136,137] This autocrine stimulation reinforces the myofibroblast phenotype and drives other fibroblasts to transdifferentiate too. [135,136] This autocrine TGF-b1 signaling also augments collagen I production from the myofibroblast. The new collagen I fibers are deposited in bundles that align with the direction of myofibroblast contractile force, thus strengthening the tissue and reinforcing it to resist mechanical shear stress.[133-136] At this stage of wound healing, myofibroblasts begin to degrade the provisional collagen III matrix primarily through the action of matrix metalloproteinases, and this marks the transition from proliferation to the remodeling phase where the granulation tissue will mature into a scar. [123,125,133]

Remodeling:

An indent to match formatting of prior sections Remodeling or scar formation occurs in three key steps.

1.Replacement of type 3 collagen with type 1 collagen.

2.Re-alignment of type 1 collagen fibers along the myofibroblast axis.

3.Resorption of excess neuro-vascular networks and myofibroblasts restores the tissue to function.

Remodeling is the last phase of wound healing and occurs from day 21 up to one year or longer after injury.[39,40,45-47] During the remodeling phase, formation of new granulation tissue ceases as fibroblasts either die through apoptosis or differentiate into myofibroblasts. The failure to properly transition from the proliferation phase may lead to excessive or hypertrophic scarring. [46,49,123,131] Resorption of the vascular capillary network and its associated neural network are hallmarks of a mature scar tissue. Thus, the completely remodeled and fully mature scar tissue is relatively acellular and avascular.[138]

The remodeling phase primarily involves the refinement of collagen and its associated extracellular matrix. At this stage of healing collagen synthesis and destruction both occur at a greater rate compared with normal tissue.[133,134,136,138] Under normal physiological conditions, the maturing scar is a very dynamic system where the balance of anabolic and catabolic processes ultimately favors the maturation of the scar into a functional connective tissue. The normal outcome of the wound healing cascade is a functional tissue, a mature scar will have

around 80% of the strength of the original tissue.[46,49,133,138]

KEY CONCEPT: None of the components of whole blood functions alone in the normal physiology of wound healing. Cellular co-operativity is important for execution at each phase of the healing cascade.

Conclusions:

Physiology is important. The physiology of wound healing is an intricate, fine-tuned, and delicate balance of cascading cellular and molecular events that, when all goes according to plan, results in the restoration of the damaged tissue to a healthy and functional state. One key concept in the physiology of wound healing is that all of the information needed to drive the repair of an injured site is contained within the wound and the blood that enters it.[39,40,45-47,131,133,134,136,138] It is important to note that all of the components of blood; plasma, platelets, RBCs and WBCs; have important roles in tissue repair, and none of these components functions alone in the normal physiology of wound healing.[39,40,45-47,131] This foundational concept of cellular co-operativity in wound healing is believed to apply to PRP therapy as well.[29,30,44,76,112,139]

Platelet rich plasma can be prepared using various methods that will either maintain physiological ratios of blood components (leukocyte rich PRP containing RBCs), or remove specific components such as RBCs and WBCs (leukocyte poor PRP), or even just RBCs (Pure PRP). A considerable amount is known about how blood components contribute to normal wound healing however, very little is actually known about how PRP may influence this cascade of signaling events. Historically, platelets have been considered as the active component in PRP, as these are the most abundant of the blood cells that contain storage granules for growth factors and immunomodulators. However, it has been demonstrated that platelet function is compromised when other blood components are missing from PRP.[76] Therefore, it seems logical to conclude that the complete physiological repertoire of blood components, rather than any individual part, should be considered the true "active component" of PRP.[76] Until clinical studies advance to the level of sophistication that allows for the elucidation of an "optimal formulation" of PRP, maintaining the physiological context of platelets with respect to other blood cells may be one way to ensure robust and balanced PRP activity. This may indeed represent the optimal means for harvesting the efficient wound healing potential of blood, which has evolved over many millennia.

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Declarations

Conflict of Interest: WRP and BR are employees of DePuySynthes. WRP is a co-inventor on patent applications regarding the preparation and use of blood components (US 9,555,171 and US 13/250,086)

Disclaimer

The views expressed in this review article are the scientific position of the Authors and do not represent an official position of DePuy Synthes Mitek Sports Medicine or the Johnson and Johnson Family of Companies.

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