In vitro investigation of the therapeutic potential of canine platelet lysate in wound healing

by

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Abstract

Wound healing is a complex multicellular process characterized by different phases that are mediated by a combination of cascades, such as mediators and cellular components, and requires multiple cytokines and growth factors. During the inflammatory phase of the wound healing, platelets influx to the injury site to form the hemostatic plug and release cytokines and growth factors through degranulation in the wound area. These factors promote the migration and chemotaxis of inflammatory cells into the wound, enhance tissue regeneration, and synthesis and deposition of extracellular matrix. Recent efforts have been focused on the development of platelet-derived products, such as platelet lysate as an economical source of growth factors, cytokines, chemokines, and osteoconductive proteins for wound healing and tissue regeneration. Our first aim was to evaluate how different methods of preparation used to manufacture various platelet-derived formulations affect the growth and cytokine concentration of the final product. For our studies, we generated and assessed different manual methods to prepare canine platelet lysate with variable contents of leukocytes, plasma, and heat-sensitive proteins. Double centrifugation of whole blood was used to generate platelet concentrates, a pure (PC, leukocyte poor) and a leukocyte rich platelet concentrate (LPC), while a portion of both products generated with each method underwent plasma removal. Following that, platelet lysate was generated via freeze-thaw cycles and a portion of the generated platelet lysate underwent complement inactivation via heat treatment. Growth factors associated with the healing process were quantified using enzyme-linked immunosorbent assay (ELISA). We showed that platelet-derived growth factor (PDGF) was significantly higher in platelet lysate that was plasma depleted, whereas vascular endothelial growth factor (VEGF) was significantly higher in heat-treated lysate groups. Furthermore,

VEGF concentration in plasma-depleted leukocyte rich platelet lysate was significantly higher than plasma-depleted pure platelet lysate. Transforming necrosis factor-α (TNF-α) concentrations were overall very low, but significantly increased following plasma depletion. These results support that platelet lysate preparation and processing can affect growth factors and cytokine release. Our second goal was to investigate how different concentrations of various formulations of canine PL, with or without plasma proteins and heat-sensitive proteins, affect major activities of the regenerative process, such as the viability, chemotaxis, and migration of canine keratinocytes *in vitro*. We found that the keratinocytes maintained viability for up to 48 hours following the addition of various lysate formulations while a cytotoxic effect was not encountered. In addition, we found that lysates rich in plasma and content from platelets are essential for an improved chemotactic and migration activity of canine keratinocytes while plasma components alone do not seem to have a chemoattractant effect on keratinocytes. Our findings suggest that different formulations of canine platelet lysate affect the growth factor, cytokine release, and *in vitro* tissue regeneration activities, such as viability, chemotaxis, and scratch closure rate of canine keratinocytes. In conclusion, our study further supports the therapeutical potential of platelet lysate for the therapeutic management of canine wounds, as it might enhance tissue regeneration with a shortened recovery time.

Dedication

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ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblastic Growth Factor
CTGF	Connective Tissue Growth Factor
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
FGF	Fibroblast Growth Factor
HBOT	Hyperbaric Oxygen Therapy
HB-EGF	Heparin Binding Epidermal Growth Factor
HCT	Hematocrit
hLPL	Heat Treated Leukocyte Rich Platelet Lysate
hLPPL	Heat Treated Leukocyte Rich Platelet Pellet Lysate
hPL	Heat Treated Pure Platelet Lysate
hPPL	Heat Treated Platelet Pellet Lysate
IGF	Insulin-like Growth Factor
LPC	Leukocyte Rich Platelet Concentration
LPL	Leukocyte Rich Platelet Lysate
LPPC	Leukocyte Rich Platelet Pellet Concentration
LPPL	Leukocyte Rich Platelet Pellet Lysate

L-PRP	Leukocyte and Platelet Rich Plasma
LLLT	Low-Level Laser Therapy
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cells
NPWT	Negative Pressure Wound Therapy
NSAID	Nonsteroidal Anti-Inflammatory Drug
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PC	Pure Platelet Concentrate
PL	Pure Platelet Lysate
PLT	Platelet Concentration
PPC	Platelet Pellet Concentrate
PPL	Platelet Pellet Lysate
PPP	Platelet Poor Plasma
PRF	Platelet-Rich Fibrin
PRP	Platelet-Rich Plasma
RBC	Red Blood Cell
RCF	Relative Central Centrifuge Force
TIMP	Tissue Inhibitors of Metalloproteinase
TNF-α	Transforming Necrosis Factor-α
TNF-β	Transforming Necrosis Factor-β
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell

CHAPTER 1

LITERATURE REVIEW

1.1. Wound Healing

Wound results from damage in the surface epithelium or underlying connective tissue secondary to chemical, mechanical, or thermal injury [1]. Wound healing initiates immediately after a cellular barrier injury and follows a pattern of re-establishing an epithelial shield, recovering the tissue integrity, strength, and function [2], and helping prevent the entry of infectious organisms [1]. This complex process is mediated by a combination of cascades, involving mediators and cellular components, and requires multiple cytokines, and growth factors for different inhibitory and stimulatory functions [3, 4]. These include molecular interactions associated with the recruitment of mesenchymal cells, proliferation, and regeneration of the extracellular matrix [4, 5], coordinated functions of inflammatory cells, endothelial cells, fibroblasts, and keratinocytes to restore skin integrity [3, 6]. The dynamic response restores the architecture and function of the wound comprises four distinct and synchronized phases that overlap in time including hemostasis, inflammation, proliferation, and dermal remodeling [2, 7-9].

1.1.1. Phases of Wound Healing

1.1.1.1. Hemostasis

The key player in hemostasis and coagulation, platelets, become activated when they encounter the vascular subendothelial matrix and adhere to the vessel via platelet receptors. Subsequently, thrombin further triggers platelet activation, induces platelet conformation change, and releases platelet granules containing bioactive molecules that reinforce coagulation and formation of a clot [9]. The clot consists of platelets embedded in a mesh of

cross-linked fibrin fibers derived by thrombin cleavage of fibrinogen with plasma fibronectin, vitronectin, and thrombospondin [1]. The clot acts as a temporary barrier to plug the wound, helps defend against bacterial spread, provides a scaffold for immune cells, cell adhesion and endothelial cell migration, and serves as a reservoir of cytokines and growth factors that are released when platelets degranulate, stimulating early wound repair [1, 9]. Once sufficient clotting has occurred, the coagulation is regulated and inhibited to prevent excessive thrombosis. Simultaneously, smooth muscle and endothelial cells repair the damaged vessel wall in response to the released platelet-derived growth factor (PDGF) [9].

1.1.1.2. Inflammation

The inflammatory phase is characterized by the migration of neutrophils and monocytes from the blood into wound sites [1, 2, 10] by numerous chemoattractants supplied by the coagulation and activated complement pathways [1, 2]. These cells primarily help in clearing bacteria and debris, but also lay the background for keratinocyte proliferation to restore skin architecture [1]. This phase is influenced by numerous intrinsic and extrinsic factors. However, uncontrolled and exorbitant inflammation can lead to tissue damage and delay the healing process. Thus, the immune cell response should be contingent, with the appropriate response to infection to allow wound resolution [9].

The neutrophils are recruited to the site of injury by many mediators [9] and are found at the site of injury within minutes after the insult [1]. Their primary goal is to destroy and remove necrotic tissue and pathogens [2, 9]. In non-infected wounds, neutrophils usually decrease within days after injury occurs [9].

Circulating monocytes migrate into the wound tissue and differentiate into macrophages [9], and they are the most abundant hematopoietic cells in intact skin [1]. The

degree of macrophage infiltration varies per species, however, in normally healing wounds, the macrophages remain for days to weeks [2]. Macrophages are the master effector cells in tissue repair [9], as they carry out debridement, microbial killing, and coordinate the later phases of repair. Furthermore, activated macrophages contain the ability to synthesize and secrete cytokines to recruit and activate cells involved in fibroplasia, angiogenesis, and epithelization [2].

1.1.1.3. Proliferation

The proliferative phase initiates before the complete resolution of the inflammatory phase [2] and gears toward closing the excisional skin wound [1]. This phase involves the activation of keratinocytes, fibroblasts, macrophages, and endothelial cells to coordinate wound closure, matrix deposition, and angiogenesis [9]. The macrophages produce mediators stimulating angiogenesis and fibroplasia; the fibroblasts proliferate and synthesize the extracellular matrix (ECM); the new blood vessels carry nutrients and oxygen needed for cell growth [2].

Within hours of the wound insult, several mediators, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding epidermal growth factor (HB-EGF), and fibroblast growth factor (FGF) stimulate migration and proliferation of keratinocytes from the wound edge as well as epithelial cells from the hair follicles [1]. Epithelization occurs until the wound is covered and the migration ceases. The epithelization is more rapid in superficial wounds, however, the rate of wound closure depends on several factors, including the species, wound site, substrate, and size [2].

Fibroblasts contribute to replacing provisional fibrin-rich matrix with more substantial granulation tissue [9]. The proliferation and migration of fibroblast are signaled by several

chemoattractants, cytokines, and growth factors including TGF-α, PDGF, EGF, transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), and fibroblast growth factor (FGF) [2].

Another component of proliferation is angiogenesis. Angiogenesis is the process of forming new blood vessels, and it is triggered by hypoxia [9]. Pro-angiogenic factors such as TGF-β [11], FGF, and vascular endothelial growth factor (VEGF) [2, 9, 11] stimulate microvascular endothelial cells to proliferate and migrate into the wound bed, where they sprout new vessels that merge with other to develop a tubular network [2, 9].

1.1.1.4. Matrix remodeling

The tissue remodeling phase is characterized by wound contraction which is attributed to the myofibroblasts [2] that alleviate epithelization and scar formation. Fibroblasts are the primary cells responsible for ECM remodeling in wounds. Through the simulation of TGF-β, they differentiate into myofibroblasts, which are crucial for wound contraction [1]. The remodeling of the ECM begins with the initial formation of the fibrin clot and culminates years later with the formation of the mature type I collagen, which provides the scar with greater tensile strength [9]. This process requires a balance between collagen breakdown and synthesis, which is facilitated by the matrix metalloproteinase (MMP) [2, 9], and their natural inhibitors named as tissue inhibitors of metalloproteinase (TIMP). In case of any discrepancy balance between MMPs and TIMPs can lead to abnormal resolution and delayed repair [2].

1.1.2. Growth Factors and Wound Healing

During the inflammatory phase of wound healing, platelets play a crucial role by initiating the clotting process to form a hemostatic plug and releasing cytokines and growth factors at the wound site [11]. These factors attract inflammatory cells to the wound, which

help in bacteria killing, but also contribute to the synthesis of additional growth factors, and stimulate angiogenesis and matrix production [11]. Cytokines and growth factors from platelets (EGF, TGF-β) and macrophages stimulate keratinocyte migration across the granulation tissue, leading to epithelialization [11-13]. Overall, these growth factors expedite wound healing by reducing inflammatory molecules released from keratinocytes and macrophages and regulating tissue remodeling [11-13].

1.1.3. Chronic Wound

When the normal repair response is disrupted and there is no healing within 6 weeks, it is termed a chronic wound. It can lead to two different outcomes: an ulcerative skin defect, known as a chronic wound, or an excessive formation of scar tissue such as a hypertrophic scar or keloid [14]. Chronic wounds are characterized by high numbers of Langerhans cells, neutrophils, pro-inflammatory macrophages, and proteases [9]. Its hallmark is persistent inflammation, however, some of the important functions of the immune cells may be also disrupted including bactericidal and phagocytic activity [14]. Recently, the presence and persistence of wound infections, particularly those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have been recognized as major factors contributing to chronic wounds [9].

Although simplified below, it is important to highlight that the causes of delay in healing are often multifactorial and complex. It is affected by local and systemic defects, including hormone imbalance, cytokines, and growth factors [9]. Many wound-related factors can affect the wound healing process, including the anatomic location of the injury, the type and the extent of tissue damage, infection, and the presence of foreign debris. Moreover,

differences in patient-related factors also influence the healing process and include the patient's age, preexisting medical conditions, and history of medications and treatments [11].

In the case of aging, the function and morphology of the skin are changed due to intrinsic factors such as hormone levels and extrinsic factors such as sun exposure [1]. There is also a reduction of dermal thickness and decreased microcirculation as aging process [11]. Several phases of the wound can be affected by aging, including the hemostasis phase, given that the platelet aggregation and degranulation are enhanced; the inflammatory phase due to increased neutrophil response, delayed monocyte infiltration, and potential decreased phagocytic activity of wound macrophages in some species. All these together can result in slower reepithelization, angiogenesis, and granulation tissue formation in elderly individuals [1].

Common metabolic diseases seen in animals include diabetes mellitus, hyperadrenocorticism, liver and kidney disease, and malnutrition in cancer animals [11]. In both aged and diabetic animals, there is a gradual loss of dermal matrix and increased susceptibility to friction damage [9]. Although clear causation for all the metabolic diseases hasn't been yet identified, it may be due to the decreased inflammatory response to the wound, impairing the chemotaxis of inflammatory cells to the site of injury and reducing bacterial concentration [11].

In veterinary medicine, the most common medication that is known to delay wound healing is glucocorticoids [11]. Although time and dose-dependent, steroids cause epidermal and granulation tissue atrophy, impair macrophage activity, reduce fibroblast proliferation, and diminish collagen synthesis; all of these can cause a delay in the healing process and reduce wound tensile and strength [10, 11]. Another medication commonly used, the nonsteroidal

anti-inflammatory drug (NSAID) has been shown to suppress the inflammatory phase of the wound [11]. Lastly, chemotherapeutic agents and radiation therapy delay healing due to their mechanisms of action [10, 11].

1.1.3.1. Current approaches for wound treatment

An exhaustive discussion of the current therapies and mechanism of action is beyond the scope of this thesis but can be found in other references [10, 11, 15] but a brief synopsis follows. Wound care in veterinary medicine includes negative pressure wound therapy (NPWT) [10, 11], contact layer with adherent dressings (wet-dry and dry-dry) [10], semi occlusive-occlusive adherent dressings (alginate, hydrogel, and hydrocolloid), hyperosmotic agents (sugar, and honey) [10, 15], bioelectric dressing, fish skin grafts (tilapia and cod), low-level laser therapy (LLLT), photobiomodulation, and hypebaric oxygen therapy (HBOT). Moreover, regenerative medicine biologicals including mesenchymal stem cell (MSC) therapy, and platelet based approaches have been recently employed for the treatment of wounds in veterinary patients [11]. The characteristics of the wound and patient-related factors can contribute to the delay of the reparative process, leading to long healing times and extensive costs. Conventional treatments can be ineffective in some cases [6], therefore, recent efforts have been focused on the development of alternative platelet-derived products as a convenient and economical method to deliver a cocktail of growth factors that can promote wound healing.

1.2. Platelets

Platelets are discoid-shaped anucleic cell fragments and derive from megakaryocytes of bone marrow [16, 17]. They contain different types of secretory granules, including dense granules, lysosomes, and α-granules. To release the granules, platelets need to be activated.

Platelet activation is marked by changes in platelet shape change, adhesion, aggregation, and granule secretion. Initially, platelets undergo a shape change that significantly increases their surface area. Next, they adhere to the injury site and aggregate by binding to fibrinogen. Finally, the platelets release granules containing soluble factors, which recruit additional platelets to the site of injury and promote further aggregation [16].

Through the granules, platelets provide extensive functions beyond hemostasis, including initiation and propagation of the inflammatory process [17], anti-inflammatory effects [18, 19], analgesic potential [20], role in tissue repair, wound remodeling, and antimicrobial host defense [1, 16]. The dense granules promote additional platelet recruitment, aggregation, and fibrin formation [1], and contain platelet activation mediators, including adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, calcium, epinephrine, and histamine. The lysosomes store proteolytic enzymes, elastase, collagenase, and cathepsin, among others [16, 21]. Lastly, α-granules are the largest and most abundant of the platelet granules [17]. The α-granules store a series of potent bioactive mediations, including coagulation factors, immunologic and adhesion molecules, regulators of angiogenesis, and various chemokines and growth factors [21] in the inactive form [18]. Platelets are the principal source of growth factors and stimulate other cells to migrate to the area of trauma facilitating the healing process. More specifically, platelets release several growth factors that are essential for the initiation of healing, such as PDGF, TGF-β, VEGF, EGF, IGF, basic fibroblastic growth factor (bFGF), brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), attachment factors, and enzymes [16, 21, 22].

Growth factors present in the α-granules such as PDGF and VEGF are important modulators of the physiological process of wound healing [18, 23] since they can enhance

tissue regeneration via cell proliferation [3], mesenchymal cell recruitment [24] synthesis and deposition of ECM, promotion of vascular ingrowth, and angiogenesis [3, 24]. Although the use of recombinant growth factors for wound healing has been previously assessed in human medicine, the results have been discouraging mostly due to the inability to deliver a cocktail containing a variety of growth factors known to influence wound healing [25]. This along with their increased cost and limited shelf availability for their routine clinical use further limits their clinical use in veterinary medicine [3, 26]. The enhanced comprehension of platelet function in wound healing led to the concept of using platelets as a natural source of growth factors as therapeutic tools [3]. Thus, recent efforts have focused on developing platelet-derived products as an accessible and cost-effective source of growth factors, cytokines, chemokines, and osteoconductive proteins for wound healing and tissue regeneration [23, 27-29].

1.2.1. Platelet-derived products and their clinical application

Various platelet-derived products made from a patient's whole blood can be generated in human medicine and veterinary medicine, which encompass platelet-rich plasma (PRP) [11, 30-32], leukocyte and platelet-rich plasma (L-PRP), platelet lysate [3, 6, 26, 30, 33], platelet gel [4, 30, 34], fibrin glue [30, 34], and platelet-rich fibrin [30, 35, 36].

These platelet-derived products have been widely used in human medicine, with applications in dentistry [37], orthopedic surgery, cardiac surgery, plastic surgery [21, 23], ophthalmology [23], and reproductive medicine [38, 39]. Platelet-derived products are also increasingly being used in veterinary medicine. For example, canine platelet-derived products have been used to manage several conditions, including treatment of musculoskeletal conditions such as posttraumatic osteoarthritis [40, 41], tendon, and ligament injuries [41],

wound healing [7, 31, 32, 36, 42], lumbosacral stenosis, corneal ulcer, and aural hematoma [40], dentistry [34].

1.2.2. Generation of platelet concentrates

Canine platelet concentrates can be either prepared from anticoagulated whole blood following manual centrifugation, including the buffy-coat and tube methods, or can be obtained by plateletpheresis technology [22, 23, 27, 40, 43, 44], or via various commercially available systems that use centrifugation or filtration methods [22, 43, 44]. These different methods vary with regard to the blood volume, the use of anticoagulant, the centrifugation cycle and spin, and the plasma volume which can affect the final product [45].

In human medicine, plateletpheresis is the most employed method to produce platelet concentrate [46-49] and has been proven feasible and safe in dogs [49, 50] and horses [29, 51, 52]. Apheresis is a closed disposable system [27] that uses centrifugal technology to separate whole blood into its different components, collecting the platelets and returning the remaining components to the donor. Thus, this technique does not deprive the donor of blood components, besides platelets, and is potentially a safer and more efficient method compared to whole blood collection [21, 51]. In addition, plateletpheresis leads results in a lower leukocyte contamination [21]. However, the retail unit and disposable costs are greater with the apheresis method. In addition, the setup and processing times are longer than performing the buffy-coat method [27].

The manual centrifugation method for isolating and concentrating platelets is the most cost-effective with minimal equipment and technical requirements [23, 27], but extreme care should be taken when using this technique to avoid bacterial contamination [27]. The manual centrifugation method has great variability in the manufacturing process including the whole

blood volume, the inclusion or exclusion of leukocytes [53], and speed and centrifugation cycles [27, 35]. The first centrifugation, the “soft spin”, aims to separate the blood into three different layers: red blood cells, buffy coat, and plasma (PPP, platelet-poor plasma) [27, 35, 53] based on the cell’s density gradient [23]. The red blood cell (RBC) is the heaviest cell, which will settle at the bottom, followed by the white blood cell (WBC), and lastly, the platelets which is the lightest cell. However, the cell’s specific gravity slightly overlaps, limiting the preparation of platelet concentrate free of other cell types [23, 54]. Following, the subsequent procedure differs across protocols, but the main goal is to remove the RBC and the PPP layers [35]. The second centrifugation, the “hard spin”, is designated to separate platelets from plasma and further concentrate the platelets [23, 27, 53]. Therefore, the variation in the relative central centrifuge force (RCF) and time needs to be appropriate for the correct separation of the blood components and adequate platelet recovery [23].

The activation of platelets triggers the release of their contents. Various methods have been employed to induce the release of their granule content and therefore the release of growth factor and other bioactive molecules. The methods include repeated freeze/thaw cycle, serum conversion by the addition of thrombin or calcium chloride [21, 55], sonication at different frequencies, or solvent/detergent treatment [21]. The serum conversation led to thrombin generation, fibrin formation, and platelet degranulation. However, a drawback of this technique is the addition of extraneous substances. The freeze-thaw cycle is an attractive technique because it is efficient, economical [21], and eliminates the addition of any exogenous degranulation agents [56]. A recent study has shown that the process implemented for platelet lysis via the freeze/thaw method provides optimal release of growth factors at three to five

freeze-thaw cycles [57]. In addition, platelet lysis has been demonstrated to result in higher growth factor release than activation [41].

The American Red Cross policy for platelet-rich plasma (PRP) products is defined as those containing 1,000 x 10^3 platelets μL^{-1}. In addition, accumulated data suggests that the PRP products must achieve a minimum platelet count of 300 x 10^3 platelets μL^{-1} to exhibit a therapeutic effect, which is a 4- to 5-fold higher concentration compared to the whole blood [23, 27, 35].

1.2.2.1.Variables that affect the properties of platelet-derived products

The role of the leukocytes in the effectiveness of the platelet concentrate remains controversial and is still being studied. Some studies have shown that a high concentration of leukocytes in platelet-derived products has been linked to impeding wound healing and tissue regeneration, increased scar tissue, and collagen degradation ex vivo [23, 45, 58], and contributes to the high expression of inflammatory cytokines [45, 53]. Additionally, the presence of pro-inflammatory mediators such as neutral proteases and acid hydrolases in the leukocytes, and thromboxane release from platelets may impede tissue regeneration [58]. More recently, it was found that platelet-derived products with high platelet concentrations without leukocytes displayed the highest antimicrobial activity *in vitro* [41]. On the contrary, other studies have suggested that increased leukocyte content can increase growth factor secretion [18, 59, 60], and potentially contribute to bacterial killing of contaminated wounds through the release of myeloperoxidase [61].

Standard platelet-derived biologicals used in clinical applications also contain platelet proteomes, plasma proteins including fibrinogen, fibrin, and, proteolytic and thrombogenic enzymes [56]. These factors have the potential to impact its therapeutic effects. Fibrinogen for

example is a 340-kDA acute-phase protein that mediates many inflammatory processes [34, 62]. Fibrinogen and fibrin have well described roles in hemostasis and can dose-dependently increase mesenchymal stromal cells' secretion of pro-inflammatory cytokines [62]. Its presence can be beneficial for both hard and soft tissue healing by converting it into a potent fibrin-based scaffold capable of triggering cell colonization and tissue regeneration [63]. However, accumulation of fibrin in injured tissue can result in significant outcomes, including persistent inflammation that may contribute to the formation of scar tissue and ulceration [64]. Previous studies have shown that plasma removal via replacement with phosphate buffered saline (PBS) depletes those detrimental factors for tissue regeneration, such as fibrinogen and procoagulant enzymes. Specifically, the presence of fibrinogen in platelet products can lead to excessive activation of the coagulation cascade and gel formation [54]. In addition, fibrinogen and its cleavage products are known for their capability of altering vasoconstriction, angiogenesis, cell migration, and proliferation in fibroblasts, smooth muscle cells, and lymphocytes [62]. Complement inactivation via heat can be pursued to enhance purity and further inactive proteolytic enzymes, and remove prothrombogenic factors and unwanted residual plasma proteins to prevent the side effects associated with toxicity caused by fibrinogen deposition [63, 64].

1.2.3. Platelet lysate characteristics and advantages

There are, however, no studies directly compared two commonly used manual centrifugation methods (pure platelet lysate [leukocyte-reduced] versus leukocyte-rich) for the generation of canine platelet lysate and their different effects on growth factor and cytokine release. Additionally, there have been no studies evaluating the impact of canine platelet lysate

on keratinocyte migration, chemotaxis, and viability, with the ultimate goal of using canine platelet lysate for wound healing.

References

1. Opneja, A., S. Kapoor, and E.X. Stavrou, *Contribution of platelets, the coagulation and fibrinolytic systems to cutaneous wound healing.* Thromb Res, 2019. 179: p. 56-63.

2. Theoret, C.L., *The pathophysiology of wound repair.* Vet Clin North Am Equine Pract, 2005. 21(1): p. 1-13.

3. Barsotti, M.C., et al., *Effect of platelet lysate on human cells involved in different phases of wound healing.* PLoS One, 2013. 8(12): p. e84753.

4. Crovetti, G., et al., *Platelet gel for healing cutaneous chronic wounds.* Transfus Apher Sci, 2004. 30(2): p. 145-51.

5. Farghali, H.A., et al., *Antimicrobial action of autologous platelet-rich plasma on MRSA-infected skin wounds in dogs.* Sci Rep, 2019. 9(1): p. 12722.

6. Ranzato, E., M. Patrone, L. Mazzucco, and B. Burlando, *Platelet lysate stimulates wound repair of HaCaT keratinocytes.* Br J Dermatol, 2008. 159(3): p. 537-45.

7. Iacopetti, I., et al., *Autologous Platelet-Rich Plasma Enhances the Healing of Large Cutaneous Wounds in Dogs.* Front Vet Sci, 2020. 7: p. 575449.

8. Barrientos, S., et al., *Growth factors and cytokines in wound healing.* Wound Repair Regen, 2008. 16(5): p. 585-601.

9. Wilkinson, H.N. and M.J. Hardman, *Wound healing: cellular mechanisms and pathological outcomes.* Open Biol, 2020. 10(9): p. 200223.

10. Balsa, I.M. and W.T. Culp, *Wound Care.* Vet Clin North Am Small Anim Pract, 2015. 45(5): p. 1049-65.

11. Buote, N.J., *Updates in Wound Management and Dressings.* Vet Clin North Am Small Anim Pract, 2022. 52(2): p. 289-315.

12. Bennett, N.T. and G.S. Schultz, *Growth factors and wound healing: Part II. Role in normal and chronic wound healing.* Am J Surg, 1993. 166(1): p. 74-81.

13. Gohel, M.S., et al., *The relationship between cytokine concentrations and wound healing in chronic venous ulceration.* J Vasc Surg, 2008. 48(5): p. 1272-7.

14. Martin, P. and R. Nunan, *Cellular and molecular mechanisms of repair in acute and chronic wound healing.* Br J Dermatol, 2015. 173(2): p. 370-8.

15. Fahie, M.A. and D. Shettko, *Evidence-based wound management: a systematic review of therapeutic agents to enhance granulation and epithelialization.* Vet Clin North Am Small Anim Pract, 2007. 37(3): p. 559-77.

16. Cortese, L., P.W. Christopherson, and A. Pelagalli, *Platelet Function and Therapeutic Applications in Dogs: Current Status and Future Prospects.* Animals (Basel), 2020. 10(2).

17. Blair, P. and R. Flaumenhaft, *Platelet alpha-granules: basic biology and clinical correlates.* Blood Rev, 2009. 23(4): p. 177-89.

18. Bendinelli, P., et al., *Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF-kappaB inhibition via HGF.* J Cell Physiol, 2010. 225(3): p. 757-66.

19. Zhang, J., et al., *HGF mediates the anti-inflammatory effects of PRP on injured tendons.* PLoS One, 2013. 8(6): p. e67303.

20. Kuffler, D.P., *Variables affecting the potential efficacy of PRP in providing chronic pain relief.* J Pain Res, 2019. 12: p. 109-116.

21. Burnouf, T., D. Strunk, M.B. Koh, and K. Schallmoser, *Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation?* Biomaterials, 2016. 76: p. 371-87.

22. Carr, B.J., et al., *Canine Platelet-Rich Plasma Systems: A Prospective Analysis.* Front Vet Sci, 2015. 2: p. 73.

23. Shin, H.S., H.M. Woo, and B.J. Kang, *Optimisation of a double-centrifugation method for preparation of canine platelet-rich plasma.* BMC Vet Res, 2017. 13(1): p. 198.

24. Tambella, A.M., et al., *Autologous platelet gel to treat chronic decubital ulcers: a randomized, blind controlled clinical trial in dogs.* Vet Surg, 2014. 43(6): p. 726-33.

25. Robson, M.C., T.A. Mustoe, and T.K. Hunt, *The future of recombinant growth factors in wound healing.* Am J Surg, 1998. 176(2A Suppl): p. 80S-82S.

26. Jafar, H., et al., *Platelet lysate promotes the healing of long-standing diabetic foot ulcers: A report of two cases and in vitro study.* Heliyon, 2020. 6(5): p. e03929.

27. Perazzi, A., et al., *Description of a double centrifugation tube method for concentrating canine platelets.* BMC Vet Res, 2013. 9: p. 146.

28. Kaushik, A. and M.S. Kumaran, *Platelet-Rich Plasma: The Journey so Far !* Indian Dermatol Online J, 2020. 11(5): p. 685-692.

29. Gordon, J., S. Alvarez-Narvaez, and J.F. Peroni, *Antimicrobial Effects of Equine Platelet Lysate.* Front Vet Sci, 2021. 8: p. 703414.

30. Bharti, D., et al., *Therapeutic applications of canine platelets and their derivatives: a narrative review.* Top Companion Anim Med, 2024. 58: p. 100840.

31. Farghali, H.A., N.A. AbdElKader, M.S. Khattab, and H.O. AbuBakr, *Evaluation of subcutaneous infiltration of autologous platelet-rich plasma on skin-wound healing in dogs.* Biosci Rep, 2017. 37(2).

32. Jee, C.H., et al., *Effect of autologous platelet-rich plasma application on cutaneous wound healing in dogs.* J Vet Sci, 2016. 17(1): p. 79-87.

33. El Backly, R., et al., *Platelet lysate induces in vitro wound healing of human keratinocytes associated with a strong proinflammatory response.* Tissue Eng Part A, 2011. 17(13-14): p. 1787-800.

34. Attili, A.R., et al., *Antibacterial Properties of Canine Platelet-Rich Plasma and Other Non-Transfusional Hemo-Components: An in vitro Study.* Front Vet Sci, 2021. 8: p. 746809.

35. Dohan Ehrenfest, D.M., L. Rasmusson, and T. Albrektsson, *Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF).* Trends Biotechnol, 2009. 27(3): p. 158-67.

36. Soares, C.S., et al., *Management of canine wounds using platelet-rich fibrin (PRF) biomaterial. A case series report.* Vet Med Sci, 2024. 10(3): p. e1236.

37. Fan, Y., K. Perez, and H. Dym, *Clinical Uses of Platelet-Rich Fibrin in Oral and Maxillofacial Surgery.* Dent Clin North Am, 2020. 64(2): p. 291-303.

38. Sharara, F.I., et al., *A narrative review of platelet-rich plasma (PRP) in reproductive medicine.* J Assist Reprod Genet, 2021. 38(5): p. 1003-1012.

39. Vali, S., et al., *The Clinical Application of Platelet-Rich Plasma in the Female Reproductive System: A Narrative Review.* Life (Basel), 2023. 13(12).

40. Hagen, A., et al., *Platelet Lysate for Mesenchymal Stromal Cell Culture in the Canine and Equine Species: Analogous but Not the Same.* Animals (Basel), 2022. 12(2).

41. Gilbertie, J.M., et al., *Platelet-rich plasma lysate displays antibiofilm properties and restores antimicrobial activity against synovial fluid biofilms in vitro.* J Orthop Res, 2020. 38(6): p. 1365-1374.

42. Karayannopoulou, M., et al., *Effect of locally injected autologous platelet-rich plasma on second intention wound healing of acute full-thickness skin defects in dogs.* Vet Comp Orthop Traumatol, 2015. 28(3): p. 172-8.

43. Franklin, S.P., et al., *Influence of Cellular Composition and Exogenous Activation on Growth Factor and Cytokine Concentrations in Canine Platelet-Rich Plasmas.* Front Vet Sci, 2017. 4: p. 40.

44. Franklin, S.P., B.C. Garner, and J.L. Cook, *Characteristics of canine platelet-rich plasma prepared with five commercially available systems.* Am J Vet Res, 2015. 76(9): p. 822-7.

45. McCarrel, T.M., T. Minas, and L.A. Fortier, *Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy.* J Bone Joint Surg Am, 2012. 94(19): p. e143(1-8).

46. Singh, R.P., N. Marwaha, P. Malhotra, and S. Dash, *Quality assessment of platelet concentrates prepared by platelet rich plasma-platelet concentrate, buffy coat poor-platelet concentrate (BC-PC) and apheresis-PC methods.* Asian J Transfus Sci, 2009. 3(2): p. 86-94.

47. Rock, G. and D.M. Sutton, *Apheresis: man versus machine.* Transfusion, 1997. 37(10): p. 993-5.

48. Amanat, S.T., et al., *Clinical Indications and Adverse Reactions of Platelet Apheresis.* J Coll Physicians Surg Pak, 2015. 25(6): p. 403-6.

49. Callan, M.B., et al., *Clinical and clinicopathologic effects of plateletpheresis on healthy donor dogs.* Transfusion, 2008. 48(10): p. 2214-21.

50. Posner, L.P., J.L. Willcox, and S.E. Suter, *Apheresis in three dogs weighing <14 kg.* Vet Anaesth Analg, 2013. 40(4): p. 403-9.

51. Sumner, S.M., et al., *Platelet lysate obtained via plateletpheresis performed in standing and awake equine donors.* Transfusion, 2017. 57(7): p. 1755-1762.

52. Naskou, M.C., et al., *Platelet lysate as a novel serum-free media supplement for the culture of equine bone marrow-derived mesenchymal stem cells.* Stem Cell Res Ther, 2018. 9(1): p. 75.

53. Arnoczky, S.P. and S. Sheibani-Rad, *The basic science of platelet-rich plasma (PRP): what clinicians need to know.* Sports Med Arthrosc Rev, 2013. 21(4): p. 180-5.

54. Araki, J., et al., *Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen.* Tissue Eng Part C Methods, 2012. 18(3): p. 176-85.

55. Textor, J.A. and F. Tablin, *Activation of equine platelet-rich plasma: comparison of methods and characterization of equine autologous thrombin.* Vet Surg, 2012. 41(7): p. 784-94.

56. Chou, M.L., et al., *Tailor-made purified human platelet lysate concentrated in neurotrophins for treatment of Parkinson's disease.* Biomaterials, 2017. 142: p. 77-89.

57. Strandberg, G., et al., *Standardizing the freeze-thaw preparation of growth factors from platelet lysate.* Transfusion, 2017. 57(4): p. 1058-1065.

58. Schnabel, L.V., et al., *Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons.* J Orthop Res, 2007. 25(2): p. 230-40.

59. Zimmermann, R., et al., *Preparation of highly concentrated and white cell-poor platelet-rich plasma by plateletpheresis.* Vox Sang, 2008. 95(1): p. 20-5.

60. Castillo, T.N., M.A. Pouliot, H.J. Kim, and J.L. Dragoo, *Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems.* Am J Sports Med, 2011. 39(2): p. 266-71.

61. Lincoln, J.A., et al., *Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages.* Infect Immun, 1995. 63(8): p. 3042-7.

62. Copland, I.B., et al., *The effect of platelet lysate fibrinogen on the functionality of MSCs in immunotherapy.* Biomaterials, 2013. 34(32): p. 7840-50.

63. Delila, L., et al., *Extensive characterization of the composition and functional activities of five preparations of human platelet lysates for dedicated clinical uses.* Platelets, 2021. 32(2): p. 259-272.

64. Widyaningrum, R., et al., *A purified human platelet pellet lysate rich in neurotrophic factors and antioxidants repairs and protects corneal endothelial cells from oxidative stress.* Biomed Pharmacother, 2021. 142: p. 112046.

65. Naskou, M.C., et al., *Innate immune responses of equine monocytes cultured in equine platelet lysate.* Vet Immunol Immunopathol, 2018. 195: p. 65-71.

66. Palombella, S., et al., *Systematic review and meta-analysis on the use of human platelet lysate for mesenchymal stem cell cultures: comparison with fetal bovine serum and considerations on the production protocol.* Stem Cell Res Ther, 2022. 13(1): p. 142.

67. Ranzato, E., et al., *Scratch wound closure of C2C12 mouse myoblasts is enhanced by human platelet lysate.* Cell Biol Int, 2009. 33(9): p. 911-7.

68. Ranzato, E., et al., *Platelet lysate modulates MMP-2 and MMP-9 expression, matrix deposition and cell-to-matrix adhesion in keratinocytes and fibroblasts.* Exp Dermatol, 2011. 20(4): p. 308-13.

CHAPTER 2

GROWTH FACTOR AND CYTOKINE CHARACTERIZATION OF MANUAL CANINE PLATELET LYSATE PREPARATIONS WITH VARIABLE LEUKOCYTE CONCENTRATIONS, PLASMA CONTENT, AND HEAT-SENSITIVE PROTEINS[1]

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Abstract

Platelet lysate is an acellular platelet product containing factors released from secretory granules, including cytokines and growth factors. This study aimed to evaluate different centrifugation methods used to prepare canine platelet lysate with variable content of leukocytes, plasma, and heat-sensitive proteins.

Whole blood was collected from six dogs and two double-spin preparation methods were used to generate the platelet concentrate, a pure (PC, leukocyte poor) and a leukocyte rich

platelet concentrate (LPC). A portion of both methods underwent plasma depletion via centrifugation and platelet lysate was generated via freeze-thaw cycles. A portion of the generated platelet lysate underwent complement inactivation via heat treatment. Growth factors (TGF-β1, VEGF, TNF-α, PDGF-BB, HGF) were quantified in all different platelet lysate preparations using ELISAs.

Both platelet concentrates had a 6.7-fold increase in platelet concentration. White blood cell (WBC) concentration compared to whole blood increased 1.2-fold times in PC and 1.9-fold times in LPC. Negligible concentration of platelets, WBC and hematocrit were identified in all lysate groups. Statistically significant differences were identified for PDGF, VEGF, and TNF-α, and not for TGF-β or HGF. No growth factor differences were noted between centrifugation methods. PDGF was significant higher in platelet lysate that was plasma depleted. VEGF was significantly higher in heat-treated lysate groups. TNF-α concentrations were overall very low, though were noted to significantly increase following plasma depletion.

These results support that growth factors and cytokine release can be affected by the platelet lysate preparation and processing.

Introduction

Platelets are non-nucleated, discoid-shaped cells that contain three different types of secretory granules, including lysosomes, dense granules, and α-granules. Through these granules, platelets provide a magnitude of functions beyond hemostasis, including initiation and propagation of the inflammatory process (1), anti-inflammatory effects (2, 3), and analgesic potential (4). The α-granules are the largest and most abundant of the platelet

granules (1), which contain and release cytokines and growth factors that affect tissue regeneration, alleviate inflammation, and influence anabolic processes responsible for the recruitment and activation of other inflammatory cells (5, 6). Platelet-derived products also exhibit antimicrobial activity, though the mechanisms are poorly understood; complement and complement binding protein present in platelet α-granules may be responsible for its antimicrobial effects (1, 7).

Growth factors present in the α-granules such as platelet-derived growth factor (PDGF) and vascular endothelial growth factors (VEGF), are important modulators of the physiological process of wound healing (3, 8) since they can enhance tissue regeneration (9), mesenchymal cell recruitment (10), synthesis and deposition of extracellular matrix (ECM), and angiogenesis (9, 10). Thus, recent practices include the administration of recombinant growth factors for wound healing. However, the rising production cost as well as limited availability precludes the clinical use of such products (9). Recent efforts have been focused on the development of platelet-derived products as an economical source of growth factors as well as cytokines, chemokines, and osteoconductive proteins for wound healing and tissue regeneration (8, 11, 12, 13).

Platelet lysate is a platelet-derived acellular product in which the platelet-derived growth factors have been released, the cell membranes removed, and thus the product can be stored for a prolonged period while offering the same benefits as platelet concentrates (14). Additionally, lysate allows wider allogeneic use, longer storage options, and can be pooled from different donors to alleviate individual donor-to-donor variability and allow optimal standardization (14, 15, 16). Platelet lysate can be generated from platelet concentrates via different methods such as plateletpheresis and manual centrifugation, including buffy-coat-

based and tube method (13, 17), and via several commercially available systems that employ centrifugation or filtration-based methods (18, 19, 20). The activation of platelets leading to the release of their granule content can be achieved with freeze/thaw cycles, thrombin or calcium chloride (21, 22), sonication at different frequencies, or solvent/detergent treatment (22).

The role of the leukocytes in the therapeutic efficiency of these products remains controversial. A few studies have shown that a high concentration of leukocytes in platelet-derived products has been linked to impeding wound healing and tissue regeneration, increased scar tissue, and collagen degradation ex vivo (8, 24, 25). Additionally, the presence of pro-inflammatory mediators such as neutral proteases and acid hydrolases in the leukocytes, and thromboxane release from platelets may impede tissue regeneration (24). On the contrary, other studies have suggested that increased leukocyte content can increase growth factor secretion (3, 26, 27), and potentially contribute to bacterial killing of contaminated wounds via the release of myeloperoxidase (28).

Other constituents within the plasma of platelet products, such as fibrin and fibrinogen, may also influence its therapeutic effects. Heat treatment of human platelet pellet lysate, that is manufactured by the lysis of isolated platelets and is plasma free, has been studied to remove fibrinogen and procoagulant and proteolytic enzymes, including complement inactivation, as well as to ensure biocompatibility and safety. Fibrin and fibrinogen have a detrimental role in the modulation of acute inflammation (29) and can dose-dependently increase mesenchymal stromal cells secretion of pro-inflammatory cytokines, including MCP-1, IL-8, and IL-6. Fibrinogen and its cleavage products are known for their capability of altering vasoconstriction, angiogenesis, cell migration and proliferation in fibroblasts, smooth muscle

cells, and lymphocytes (30). The prolonged accumulation of fibrin in injured tissue can result in significant outcomes, including persistent inflammation that may contribute to the formation of scar tissue and ulceration (29).

There are, however, no studies directly evaluating two commonly used manual centrifugation methods (pure platelet lysate [leukocyte reduced] versus leukocyte rich method) for the generation of canine platelet-derived products and their different effects on growth factor and cytokine release. The objective of this study was to evaluate how different centrifugation methods used to prepare platelet-derived products with variable leukocyte content, presence or absence of plasma proteins and heat-sensitive proteins (complement) affects final growth and cytokine concentrations. We hypothesized that the concentration of leukocytes, presence of plasma proteins and complement will affect the growth factor and cytokine concentration of canine platelet lysate.

Materials and Methods

Blood Acquisition

Six purpose bred canine blood donors (three male and three female beagles) aged 5.5-11.8 years old (median: 7.9 years), and body weights ranging from 13.5 - 38.8 kg (median: 19.40 kg) were included in this study. The health status of the donor dogs was evaluated prior to blood collection by clinical examination and evaluation of the hematocrit and plasma protein

concentration. The study was approved by Auburn University Animal Care and Use Program (IACUC).

Whole blood was withdrawn aseptically from the jugular vein using a 22 g needle. A total of 100 mL of whole blood was collected from each donor and was divided into 8 mL blood collection tubes containing acid citrate dextrose-A solution (ACD-A; 3.2 %). A small portion of blood was collected into an EDTA tube and a serum tube without a clot activator to perform a complete blood count (CBC) and serum biochemistry profile, respectively. The CBC was performed using ADVIA 2120 Hematology Analyzer (Siemens Medical Solutions, Pennsylvania, USA) and chemistry using Cobas C311 (Roche, IN, USA) at the Clinical Pathology Laboratory at Auburn University immediately after the collection. A blood smear of each donor was evaluated to confirm the results and the presence of platelet clumping.

Generation of platelet concentrates

Whole blood was divided into 10 mL aliquots that were centrifuged in 15 mL conical tubes. Blood from each donor was separated equally to generate platelet concentrates via two different methods to create pure platelet concentrate (PC) and leukocyte rich platelet concentrate (LPC). A double-spin preparation method was used for both methods.

PC, a leukocyte poor method, was manufactured as previously described (8) (Figure 1A). Briefly, blood was centrifuged at 1000 g for 5 minutes without a centrifugal break at room temperature (Sorvall X Pro / ST Plus, ThermoFisher Scientific, USA). Following the first centrifugation, the whole plasma fraction above the buffy coat was pooled and centrifuged again at 1500 g for 15 minutes with a centrifugation break.

LPC, a leukocyte rich method, was manufactured as previously described (31) (Figure 1A). Blood was centrifuged at 180 g for 20 minutes without a centrifugal break at room

temperature. Subsequently, the plasma fraction and buffy coat layer were pooled from each tube and centrifuged at 650 g for 15 minutes with a centrifugation break to produce a platelet pellet.

After the second centrifugation for both methods, the platelet-poor fraction was removed and saved in a separate tube while the platelet concentration was determined via CBC (Heska Element HT5, Heska, Colorado, USA). Subsequently, the platelet concentrates were resuspended with the appropriate amount of platelet poor plasma to achieve a final concentration of 0.8 – 1 x 106/μL, as recommended by the American Red Cross for PRP (32). An aliquot from each preparation was submitted for a chemistry profile included concentration of bicarbonate, sodium, potassium, chloride, anion gap, calcium, glucose, total protein, and albumin.

Plasma depletion of platelet concentrates

A portion of both PC and LPC that were generated as above underwent plasma depletion to remove plasma-related proteins. Specifically, platelet concentrates were centrifuged at 3000 g for 30 minutes (Fresco 17 Microcentrifuge, ThermoFisher Scientific, USA) at 22℃. The plasma was removed, saved, and the platelet pellet was gently resuspended with equal volume of sterile phosphate-buffered saline (PBS) to produce plasma-free pure platelet pellet concentrate (PPC) and leukocyte rich platelet pellet concentrate (LPPC) (Figure 1B).

Generation of platelet lysates

Lysis of platelets from all preparations (PC, LPC, PPC, LPPC) was performed by five freeze/thaw cycles in liquid nitrogen and thawing at 37℃. Following, all preparations were centrifuged at 20,000 g for 20 minutes to remove platelet membranes and other cellular debris

(Figure 1C). This process produced pure platelet lysate (PL), leukocyte rich platelet lysate (LPL), platelet pellet lysate (PPL), and leukocyte rich platelet pellet lysate (LPPL). Sample supernatants were stored frozen at -80°C until further use. To decrease individual variability, lysates within each group were pooled from three donors to generate two pooled batches of platelet lysate per method.

Heat-treatment

A portion of pooled PL, LPL, PPL, and LPPL underwent heat treatment for complement inactivation in a dry bath at 56°C for 30 minutes as previously described (29). After heat treatment, the samples were cooled on ice for at least 5 minutes and then centrifuged at 10,000 g for 15 minutes at 4°C to remove any insoluble components. This process produced the following groups: heat-treated pure platelet lysate (hPL), heat-treated leukocyte rich platelet lysate (hLPL), heat-treated pure platelet pellet lysate (hPPL), and heat-treated leukocyte rich platelet pellet lysate (hLPPL) (Figure 1D). Sample supernatants were stored frozen at -80°C until further use.

Growth factor and cytokine quantification

Growth factors and cytokines were quantified for all the above preparation using enzyme-linked immunosorbent assays (ELISAs), which were previously validated for use with canine plasma, and included: transforming growth factor-beta1 (TGF-β1) (Human, Mouse/Rat/Porcine/Canine TGF-β1 Quantikine ELISA Kit, R&D Systems, MN, USA) (14, 19, 26, 33) vascular endothelial growth factor (VEGF) (Canine VEGF Immunoassay, R&D Systems, Minneapolis, MN, USA) (33), tumor necrosis factor α (TNF-α) (Canine TNF-α Immunoassay, R&D System, Minneapolis, MN, USA) (19, 33), platelet-derived growth factor (PDGF-BB) (Canine PDGF-BB ELISA KIT, Invitrogen, ThermoFisher Scientific, CA, USA)

(8, 14, 19, 33) and hepatocyte growth factor (HGF) (Canine HGF ELISA Kit, Invitrogen, ThermoFisher Scientific, CA, USA). ELISAs were performed in duplicates and absorbance was read on a BioTek Synergy H1 multimode reader (Agilent, CA, USA) with wavelength absorption and corrected per manufacturer's instructions.

Statistical Analysis

All data were imported into a statistical analysis program (GraphPad Prism; Graphpad Software Inc. San Diego, CA, USA). Normality was assessed via the visual examination of histograms of the residual, normal plots of residuals, and by using the Shapiro-Wilks test. The equality of variances was assessed using Levene's test and plotting residuals against the fitted value. Hypotheses were tested by t-test and repeated-measures analysis of variance (ANOVA) after confirming normal distribution. Statistical significance was assessed using a 2-away analysis of variance (ANOVA). Tukey's test was used to adjust for multiple paired comparisons. For non-parametric data, the analysis was performed using a mixed effects model. All statistical analysis was performed at $p < 0.05$ level of significance. Continuous data were summarized and reported as mean ± standard deviation. All sample analyses were performed in duplicates.

Results

Hematologic values

The mean concentration of platelets, white blood cells (WBC), and hematocrit (HCT) obtained from the whole blood and each platelet concentrate are presented in Table 1. The mean (± SD) platelet concentration for whole blood was 394.3 ± 164.34 x $10^3/\mu L$, and the

WBC concentration was 8.27 ± 1.67 x10^3/μL. Both PC and LPC had a 6.7-fold increase of their platelet concentration (2,646.0 ± 751.18 x 10^3/μL and 2,661 ± 1,150.89 x 10^3/μL, respectively). The WBC concentration of PC increased 1.2-fold times compared to whole blood (10.2 ± 6.69 x10^3/μL) while LPC had a 1.9-fold times increase (15. 7 ± 17.65 x 10^3/μL) compared to whole blood. The HCT was almost negligible for both PC and LPC.

Negligible concentration of platelets, WBC and HCT were identified in all lysate preparations (PL, LPL, PPL, and LPPL).

Chemical analysis

The mean concentration of the chemical components analyzed are reported in Table 2. The concentration of bicarbonate, chloride, calcium, total protein, and albumin were statistically significantly lower in both PC and LPC compared to whole blood (Table 1). To the contrary, sodium and glucose concentrations were statistically significantly higher in PC and LPC compared to whole blood.

Growth factor concentrations

Overall, statistically significant differences among lysate groups were identified for the concentration of PDGF, VEGF, and TNF-α, while no statistically significant difference was identified for TGF-β and HGF concentrations.

PDGF concentrations among the lysate groups are depicted in Figure 2. A statistically significant difference was not identified between all groups (Figure 2A). When separated by platelet concentrate method, a statistically significantly higher concentration of PDGF production was identified for both platelet pellet lysate groups (plasma depleted groups) compared to platelet lysate groups - PPL (10599.6 ± 1676.48 pg/mL) and LPPL (10841.9 ± 3177.03 pg/mL) had higher PDGF concentrations than PL (1988.00 ± 1792.94 pg/mL) and

LPL (1349.83 ± 1231.06 pg/mL) (p = 0.043 and p = 0.037, respectively; Figure 2B and C). Nonsignificant trends noted for PDGF included an increase following complement inactivation: hPL (4310.24 ± 5263.67 pg/mL) and hLPL (2882.76 ± 3443.67 pg/mL) compared to PL and LPL (values noted above). The opposite trend was observed in plasma depleted preparations following complement inactivation. A trend for a decreased PDGF concentration was noted in hPPL (8177.14 ± 494.18 pg/mL) and hLPPL (7055.34 ± 946.02 pg/mL) compared to PPL (10841.9 ± 3177.03 pg/mL) and LPPL (10599.6 ± 1676.48 pg/mL).

VEGF concentrations are illustrated in Figure 3. VEGF concentrations were significantly higher in LPPL (50.82 ± 14.23 pg/mL) compared to PPL (37.08 ± 2.40 pg/mL, p = 0.480; Figure 3A); hPL (63.03 ± 13.57 pg/mL) compared to PL (35.00 ± 1.07 pg/mL, p = 0.006) and PPL (37.08 ± 2.40 pg/mL, p = 0.008; Figure 3B). VEGF concentrations were significantly higher in hPPL (50.90 ± 1.68 pg/mL) compared to PPL (37.08 ± 2.40 pg/mL, p = 0.047) and PL (35.00 ± 1.07 pg/mL, p = 0.032). Similarly, hLPL (62.57 ± 24.63 pg/mL) had significantly higher VEGF concentrations compared LPL (44.80 ± 9.19 pg/mL, p = 0.024; Figure 3C). Overall, a trend was noted for VEGF concentrations to be higher in the leukocyte rich preparation method, however a statistically significant difference was not identified in groups other than LPPL and PPL as mentioned.

TNF-α measurements are illustrated in Figure 4. Overall, the concentration of TNF-α was very low. No significant difference was noted when all groups were compared (Figure 4A). When groups were analyzed within each platelet concentrate method, significantly higher TNF-α concentrations were identified in PPL (6.49 ± 0.68 pg/mL) compared to PL (below detection range, p = 0.012), hPL (below detection range, p = 0.012) and hPPL (below detection range, p = 0.018; Figure 4B). Similarly, significantly higher TNF-α concentrations were

identified in LPPL (5.51 ± 1.18 pg/ml) compared to LPL (below detection range; p = 0.019), hLPL (below detection range, p = 0.019), and hLPPL (below detection range, p = 0.025; Figure 4C).

TGF-β concentrations are shown in Figure 5A. A statistically significant difference was not identified between any of the groups. A trend towards higher TGF-β concentrations was noted in PPL (158048 ± 138581 pg/mL) and LPPL (127067 ± 80984.8 pg/mL) compared to other groups, which decreased with heat/complement inactivation (hPL = 127067 ± 80984.8 pg/mL, hLPPL = 54887.7 ± 13721 pg/mL).

HGF concentrations are presented in Figure 5B. No significant difference was noted between groups. Higher HGF concentrations were measured in PL (41.7890 ± 58.4791 pg/mL) and LPL (76.9850 ± 107.982 pg/mL) compared to the remaining groups, which had low to undetectable concentrations of HGF concentration.

Discussion

In this study, we were able to show that platelet lysate produced by two different double-centrifugation methods followed by freeze/thaw cycles resulted in variable leukocyte concentration, while the presence or absence of plasma proteins, and complement or heat-sensitive factors affected growth factor and cytokine release.

In our study we used a double-spin preparation method for the generation of the various platelet concentrates. The purpose of the first centrifugation cycle is to separate the whole blood into three layers: the plasma, buffy coat, and red blood cell (13, 32) based on the cell's

density gradient (8). Platelets are the lightest cell, followed by the WBC, and lastly, the red blood cells which is the heaviest cell. However, the specific gravities of the cells slightly overlap, which limits the preparation of platelet concentrate free of other cell types by centrifugation method (8, 34). The second centrifugation is designated to separate platelets from plasma and further concentrate the platelets (8, 13). Therefore, the variation in the relative central centrifuge force (RCF) and time needs to be appropriate for the appropriate separation of the blood components and adequate platelet recovery (8).

The platelet count of the PC in this study was similar to previously reported with a 6.7-fold increase compared to whole blood, though the leukocyte concentration was higher with a 1.2-fold increase in leukocyte count compared to whole blood (8). LPC also achieved a 6.7-fold increase in platelet concentration and a 1.9-fold increase in leukocyte count, a higher platelet concentration and a similar leukocyte concentration compared to previously described concentrations by Attili et al 2021 (31). According to The American Red Cross policy, PRP products are defined as those containing 1,000 x 103 platelets μL-1 and accumulated data suggests that the PRP products must achieve a minimum platelet count of 300 x 103 platelets μL-1 to exhibit a therapeutic effect. (8, 13, 32). In our study, both methods achieved the guidelines of The American Red Cross and recommendations of other articles with the associated number of platelets. Because both methods produce a high yield of platelets, the final platelet concentration was adjusted to 1,000 x 103/ul for the rest of the comparisons.

Our chemical analysis showed statistically significant differences between both platelet concentrate preparations (PC and LPC) and whole blood. It is likely that the release of granule contents and/or presence of leukocytes are responsible for the observed changes. The presence of ACD-A as an anticoagulant, which is commonly used for the preparation of platelet rich

products, likely contributed to the chemical analyte variation compared to whole blood that was collected with EDTA as an anticoagulant.

Beyond centrifugation methods, this study investigated the effects of plasma removal and complement inactivation on growth factor and cytokine concentrations. It has been previously demonstrated that plasma removal allows for the depletion of detrimental factors for tissue regeneration, such as fibrinogen and procoagulant enzymes (29) via replacement of the plasma with PBS. Specifically, the presence of fibrinogen in platelet products can lead to excessive activation of the coagulation cascade and gel formation (34) and has a detrimental role in acute inflammation (29). Complement inactivation was pursued to further inactive proteolytic enzymes, remove pro-thrombotic factors and unwanted plasma proteins to prevent the side effects associated with toxicity caused by fibrinogen deposition (29, 35).

In this study, plasma removal resulted in highest concentration of PDGF-BB when measured in both PPL and LPPL compared to PL and LPL. The removal of plasma and replacement with PBS, which included an extra centrifugation cycle, may have caused further platelet degranulation and release of PDGF. Following complement inactivation, PDGF-BB concentrations had a nonsignificant trend to increase in hPL and hLPL compared to PL and LPL respectively, but decreased when complement was inactivated in hPPL and hLPPL derived products. While this was not a significant difference in this study, one previous study demonstrated a decreased PDGF-AB concentration following complement inactivation made from platelets only (PPL) (35). However, Chou et al. found no significance difference in PDGF-AB concentrations after complement inactivation of both PL and PPL (36).

On the other hand, heat treatment for complement inactivation resulted in a higher concentration of VEGF in both hPL and hPPL. A similar effect was noted for hLPL and

hLPPL. This finding was in accordance with the study performed by Chou et al., (36) where higher concentrations of VEGF were identified following heat treatment of PPL. However, these results conflict with those previously reported in which a decreased concentration of VEGF was identified following heat treatment for complement inactivation in PPL (35). It is known that hypoxia stimulates the production of hypoxia-inducible factor 1α (HIF-1α), which can trigger the production of VEGF and other proangiogenic factors, including PDGF and TGF-β (37). It is possible that during our manufacturing process, a hypoxic environment was induced to the platelets resulting in a further release of VEGF. Another possibility is that growth factor analysis was performed in frozen samples, and it is possible that the plastic surface of the storage tubes had an effect on growth factor concentration. Moreover, an additive solution was not added to the products to improve stability, and this needs to be further investigated.

Studies conducted in human derived platelet products reported a higher concentration of growth factors such as PDGF, VEGF, TGF-β, and EGF in plasma depleted platelets products (PPL) (35, 36). In this study, this effect was encountered for PDGF, TNF-α, and TGF-β, but not for VEGF or HGF. Moreover, heat treatment for complement inactivation has been found to reduce the concentration of the above-mentioned growth factors except for TGF-β (35). Other studies did not encounter such an effect following heat treatment of the samples with the exception of HGF, fibroblast derived growth factor (FGF) and brain derived neurotrophic factor (BDNF) (36). Moreover, the above studies were performed in human derived platelet product, and it is possible that canine platelet characteristics and *in vitro* modification affects growth factor release differently.

The VEGF concentration in LPPL was significantly higher than PPL. It has been demonstrated that the presence of leukocytes can affect the release of growth factors from platelets (38). Castillo et al. found higher concentrations of PDGF-AB, PDGF-BB, and VEGF in leukocyte rich PRP compared to leukocyte poor PRP. However, the concentrations were not evaluated in plasma depleted products (27). Thus, it is possible that the presence of the leukocytes in the final product can affect growth factor release. However, note that the overall mean concentration of leukocytes between the two platelet concentrate methods did not strongly differ and plasma depletion was not previously evaluated by Castillo et al. (27). Future studies should focus on the production of platelet-derived products with higher leukocyte concentration and the exact effect on growth factor and chemokine release from canine platelets.

TNF-α, which is an inflammatory cytokine, was evaluated to assess whether the mode of platelet lysate production might cause a spontaneous inflammatory process. Overall, the concentration of TNF-α was very low among all groups. However, a statistically significantly higher TNF-α concentration was noted in PPL and LPPL compared to the rest of the groups. Heat inactivation resulting in a decreased concentration of TNF-α as seen in hPPL and hLPPL, but this difference was not statistically significant. It is worth mentioning that TNF-α concentrations were minimal or absent in lysate samples that were not further manipulated by plasma removal, while heat treatment did not seem to affect the concentration of TNF-α. This is in accordance to previous studies that found that canine PRP products that did not undergo activation, had minimal or absence concentrations of TNF-α (18). The degree of platelet activation was not quantified in this study and future studies should evaluate the expression of CD62P as a platelet activation marker.

Different modes of platelet lysate preparation produced variable concentrations of TGF- β with no statistical significance. However, a trend was noted for plasma depletion to increase the concentrations of TGF- β while following heat treatment decreased TGF-β concentrations. As mentioned above, a similar finding has been previously reported (35, 36) where TGF-β increased in plasma depleted products however both studies found an increased concentration of TGF-β following heat treatment (35, 36). In this study, ELISA kit that detects activated TGF-β1 were used, and thus it is possible that heat treatment inhibited further platelet activation and/or accurate detection of the growth factor of interest via our method of detection. Furthermore, this study employed a freeze/thaw process for platelet activation and no exogenous substances such as thrombin or calcium chloride were added to the products. Platelet activation method may have played a role in final growth factor concentration as well as its variation with additional processing such as plasma depletion and heat treatments. The process implemented for platelet lysis via a five-cycle (36) freeze/thaw procedure was recently shown to provide optimal release of growth factors (39). Most crucial, the freeze/thaw method eliminates the addition of any exogenous degranulation agents (36).

HGF is a mitogen for endothelial cells (40). Studies have shown that an anti-inflammatory function in tendon cells is mediated by HGF (2), and an anti-inflammatory function in chondrocytes is mediated by HGF and TNF-α (3). In addition, HGF inhibits the release of pro-inflammatory cytokines, and increases the release of IL-10 in LPS-activated macrophages (41). HGF was noted to be predominantly present in PL and LPL while a significant difference was not detected compared to other groups. A nonsignificant trend for HGF concentration to decreased following plasma depletion and heat treatment was noted.

These findings are in accordance with those previously reported in the human literature (36) where HGF statistically decreased following heat treatment of both PL and PPL.

Just as plasma and platelets play an important role in coagulation, platelet-derived products promote coagulation as well as the generation of cross-linked fibrin fibers, which is particularly important for soft tissue wound healing (42). However, this function is detrimental for other applications such as treatment for brain related disease or ocular disease (43). Thus, future studies should evaluate the effect of platelet lysate preparation on thrombin generation, thrombin proteolytic activity, presence of pro-coagulant molecules such as phosphatidylserine and activated factor XIa.

Conclusion

In summary, our results proved that growth factor and cytokine release can be affected by the mode of platelet lysate preparation and the presence of leukocytes in the final product. These differences can have a profound effect on the physiological and clinical function of platelet-derived products and such information should be carefully evaluated when developing and selecting specific platelet lysate products for clinical applications.

References

1. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009;23(4):177-89.

2. Zhang J, Middleton KK, Fu FH, Im HJ, Wang JH. HGF mediates the anti-inflammatory effects of PRP on injured tendons. PLoS One. 2013;8(6):e67303.

3. Bendinelli P, Matteucci E, Dogliotti G, Corsi MM, Banfi G, Maroni P, et al. Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF-kappaB inhibition via HGF. J Cell Physiol. 2010;225(3):757-66.

4. Kuffler DP. Variables affecting the potential efficacy of PRP in providing chronic pain relief. J Pain Res. 2019;12:109-16.

5. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008;16(5):585-601.

6. Arnoczky SP, Sheibani-Rad S. The basic science of platelet-rich plasma (PRP): what clinicians need to know. Sports Med Arthrosc Rev. 2013;21(4):180-5.

7. Radek K, Gallo R. Antimicrobial peptides: natural effectors of the innate immune system. Semin Immunopathol. 2007;29(1):27-43.

8. Shin HS, Woo HM, Kang BJ. Optimisation of a double-centrifugation method for preparation of canine platelet-rich plasma. BMC Vet Res. 2017;13(1):198.

9. Barsotti MC, Losi P, Briganti E, Sanguinetti E, Magera A, Al Kayal T, et al. Effect of platelet lysate on human cells involved in different phases of wound healing. PLoS One. 2013;8(12):e84753.

10. Tambella AM, Attili AR, Dini F, Palumbo Piccionello A, Vullo C, Serri E, et al. Autologous platelet gel to treat chronic decubital ulcers: a randomized, blind controlled clinical trial in dogs. Vet Surg. 2014;43(6):726-33.

11. Gordon J, Alvarez-Narvaez S, Peroni JF. Antimicrobial Effects of Equine Platelet Lysate. Front Vet Sci. 2021;8:703414.

12. Kaushik A, Kumaran MS. Platelet-Rich Plasma: The Journey so Far ! Indian Dermatol Online J. 2020;11(5):685-92.

13. Perazzi A, Busetto R, Martinello T, Drigo M, Pasotto D, Cian F, et al. Description of a double centrifugation tube method for concentrating canine platelets. BMC Vet Res. 2013;9:146.

14. Hagen A, Holland H, Brandt VP, Doll CU, Haussler TC, Melzer M, et al. Platelet Lysate for Mesenchymal Stromal Cell Culture in the Canine and Equine Species: Analogous but Not the Same. Animals (Basel). 2022;12(2).

15. Naskou MC, Norton NA, Copland IB, Galipeau J, Peroni JF. Innate immune responses of equine monocytes cultured in equine platelet lysate. Vet Immunol Immunopathol. 2018;195:65-71.

16. Nebie O, Devos D, Vingtdeux V, Barro L, Devedjian JC, Jonneaux A, et al. The neuroprotective activity of heat-treated human platelet lysate biomaterials manufactured from outdated pathogen-reduced (amotosalen/UVA) platelet concentrates. J Biomed Sci. 2019;26(1):89.

17. Naskou MC, Sumner SM, Chocallo A, Kemelmakher H, Thoresen M, Copland I, et al. Platelet lysate as a novel serum-free media supplement for the culture of equine bone marrow-derived mesenchymal stem cells. Stem Cell Res Ther. 2018;9(1):75.

18. Franklin SP, Garner BC, Cook JL. Characteristics of canine platelet-rich plasma prepared with five commercially available systems. Am J Vet Res. 2015;76(9):822-7.

19. Franklin SP, Birdwhistell KE, Strelchik A, Garner BC, Brainard BM. Influence of Cellular Composition and Exogenous Activation on Growth Factor and Cytokine Concentrations in Canine Platelet-Rich Plasmas. Front Vet Sci. 2017;4:40.

20. Carr BJ, Canapp SO, Jr., Mason DR, Cox C, Hess T. Canine Platelet-Rich Plasma Systems: A Prospective Analysis. Front Vet Sci. 2015;2:73.

21. Textor JA, Tablin F. Activation of equine platelet-rich plasma: comparison of methods and characterization of equine autologous thrombin. Vet Surg. 2012;41(7):784-94.

22. Burnouf T, Strunk D, Koh MB, Schallmoser K. Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? Biomaterials. 2016;76:371-87.

23. Qian Y, Han Q, Chen W, Song J, Zhao X, Ouyang Y, et al. Platelet-Rich Plasma Derived Growth Factors Contribute to Stem Cell Differentiation in Musculoskeletal Regeneration. Front Chem. 2017;5:89.

24. Schnabel LV, Mohammed HO, Miller BJ, McDermott WG, Jacobson MS, Santangelo KS, et al. Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. J Orthop Res. 2007;25(2):230-40.

25. McCarrel TM, Minas T, Fortier LA. Optimization of leukocyte concentration in platelet-rich plasma for the treatment of tendinopathy. J Bone Joint Surg Am. 2012;94(19):e143(1-8).

26. Zimmermann R, Reske S, Metzler P, Schlegel A, Ringwald J, Eckstein R. Preparation of highly concentrated and white cell-poor platelet-rich plasma by plateletpheresis. Vox Sang. 2008;95(1):20-5.

27. Castillo TN, Pouliot MA, Kim HJ, Dragoo JL. Comparison of growth factor and platelet concentration from commercial platelet-rich plasma separation systems. Am J Sports Med. 2011;39(2):266-71.

28. Lincoln JA, Lefkowitz DL, Cain T, Castro A, Mills KC, Lefkowitz SS, et al. Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. Infect Immun. 1995;63(8):3042-7.

29. Widyaningrum R, Burnouf T, Nebie O, Delila L, Wang TJ. A purified human platelet pellet lysate rich in neurotrophic factors and antioxidants repairs and protects corneal endothelial cells from oxidative stress. Biomed Pharmacother. 2021;142:112046.

30. Copland IB, Garcia MA, Waller EK, Roback JD, Galipeau J. The effect of platelet lysate fibrinogen on the functionality of MSCs in immunotherapy. Biomaterials. 2013;34(32):7840-50.

31. Attili AR, Iacoucci C, Serri E, Cuteri V, Cantalamessa A, Linardi M, et al. Antibacterial Properties of Canine Platelet-Rich Plasma and Other Non-Transfusional Hemo-Components: An in vitro Study. Front Vet Sci. 2021;8:746809.

32. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). Trends Biotechnol. 2009;27(3):158-67.

33. Birdwhistell K, Basinger R, Hayes B, Norton N, Hurley DJ, Franklin SP. Validation of commercial ELISAs for quantifying anabolic growth factors and cytokines in canine ACD-A anticoagulated plasma. J Vet Diagn Invest. 2017;29(2):143-7.

34. Araki J, Jona M, Eto H, Aoi N, Kato H, Suga H, et al. Optimized preparation method of platelet-concentrated plasma and noncoagulating platelet-derived factor concentrates: maximization of platelet concentration and removal of fibrinogen. Tissue Eng Part C Methods. 2012;18(3):176-85.

35. Delila L, Wu YW, Nebie O, Widyaningrum R, Chou ML, Devos D, et al. Extensive characterization of the composition and functional activities of five preparations of human platelet lysates for dedicated clinical uses. Platelets. 2021;32(2):259-72.

36. Chou ML, Wu JW, Gouel F, Jonneaux A, Timmerman K, Renn TY, et al. Tailor-made purified human platelet lysate concentrated in neurotrophins for treatment of Parkinson's disease. Biomaterials. 2017;142:77-89.

37. Naskou MC, Tyma JF, Gordon J, Berezny A, Kemelmakher H, Richey AC, et al. Equine Platelet Lysate Gel: A Matrix for Mesenchymal Stem Cell Delivery. Stem Cells Dev. 2022;31(17-18):569-78.

38. Zimmermann R, Arnold D, Strasser E, Ringwald J, Schlegel A, Wiltfang J, et al. Sample preparation technique and white cell content influence the detectable levels of growth factors in platelet concentrates. Vox Sang. 2003;85(4):283-9.

39. Strandberg G, Sellberg F, Sommar P, Ronaghi M, Lubenow N, Knutson F, et al. Standardizing the freeze-thaw preparation of growth factors from platelet lysate. Transfusion. 2017;57(4):1058-65.

40. Wen YH, Lin WY, Lin CJ, Sun YC, Chang PY, Wang HY, et al. Sustained or higher levels of growth factors in platelet-rich plasma during 7-day storage. Clin Chim Acta. 2018;483:89-93.

41. Coudriet GM, He J, Trucco M, Mars WM, Piganelli JD. Hepatocyte growth factor modulates interleukin-6 production in bone marrow derived macrophages: implications for inflammatory mediated diseases. PLoS One. 2010;5(11):e15384.

42. Opneja A, Kapoor S, Stavrou EX. Contribution of platelets, the coagulation and fibrinolytic systems to cutaneous wound healing. Thromb Res. 2019;179:56-63.

43. Cortes-Canteli M, Mattei L, Richards AT, Norris EH, Strickland S. Fibrin deposited in the Alzheimer's disease brain promotes neuronal degeneration. Neurobiol Aging. 2015;36(2):608-17.

Table 1: Descriptive results of hematological data presented as mean ± standard deviation from whole blood, and different platelet delivered concentrates. PLT, platelet concentration; WBC, white blood cell concentration; HTC, hematocrit; PC, pure platelet concentrate; PPC, platelet pellet concentrate; PL, pure platelet lysate; PPL, platelet pellet lysate; hPL, heat treated PL; hPPL, heat treated PPL; LPC, leukocyte rich platelet concentration; LPPC, leukocyte rich

platelet pellet concentration; LPL, leukocyte rich platelet lysate; LPPL, leukocyte rich platelet pellet lysate; hLPL, heat treated LPL; hLPPL, heat treated LPPL.

Hematology analysis			
PREPARATION	**PLT (x10^3/µL)**	**WBC (x10^3/µL)**	**HTC (%)**
Whole blood	394.3 ± 164.34	8.27 ± 1.67	51.33 ± 3.73
Pure platelet concentrates			
PC	2,646 ± 751.18	10.2 ± 6.69	0.40 ± 0.98
PPC	71.67 ± 63.09	0.04 ± 0.04	0.09 ± 0.16
PL	9.50 ± 6.36	0.03 ± 0.03	0 ± 0
PPL	1.5 ± 0.71	0 ± 0	0 ± 0
hPL	6.5 ± 4.95	0.03 ± 0.03	0 ± 0
hPPL	2.50 ± 2.12	0.01 ± 0	0 ± 0
Leukocyte rich platelet concentrates			
LPC	2,661 ± 1150.89	15.7 ± 17.65	1.80 ± 2.87
LPPC	158.0 ± 74.97	0.09 ± 0.16	0 ± 0
LPL	12 ± 1.41	0.01 ± 0	0 ± 0
LPPL	3.50 ± 3.54	0.01 ± 0	0 ± 0
hLPL	8 ± 7.07	0.06 ± 0.08	0 ± 0
hLPPL	1 ± 1.41	0 ± 0	0 ± 0

Table 2: Descriptive results of chemistry analyzed data presented as mean ± standard deviation from whole blood (n=6), and different platelet delivered concentrates. PLT, platelet concentration; WBC, white blood cell concentration; HTC, hematocrit; PC, pure platelet concentrate; LPC, leukocyte rich platelet concentrate; * denotes a significant difference compared to whole blood.

Chemistry analysis of Platelet Concentrates

Variable	WB	PC	LPC
Bicarbonate (mmol/L)	22.47 ± 2.12	5.78 ± 1.82*	6.72 ± 1.81*
Sodium (mmol/L)	145.67 ± 1.37	169.83 ± 3.19*	170.17 ± 2.64*
Potassium (mmol/L)	4.05 ± 0.21	5.45 ± 1.52	4.68 ± 1.14
Chloride (mmol/L)	107.83 ± 1.94	60.0 ± 0*	60.0 ± 0*
Calcium (mg/dL)	11.00 ± 0.51	7.43 ± 0.42*	7.63 ± 0.44*
Glucose (mg/dL)	87.00 ± 12.76	712.50 ± 46.76*	691.17 ± 25.71*
Total Protein (g/dL)	6.47 ± 0.44	4.98 ± 0.41*	4.90 ± 0.29*
Albumin (g/dL)	3.69 ± 0.36	2.91 ± 0.27*	2.93 ± 0.23*

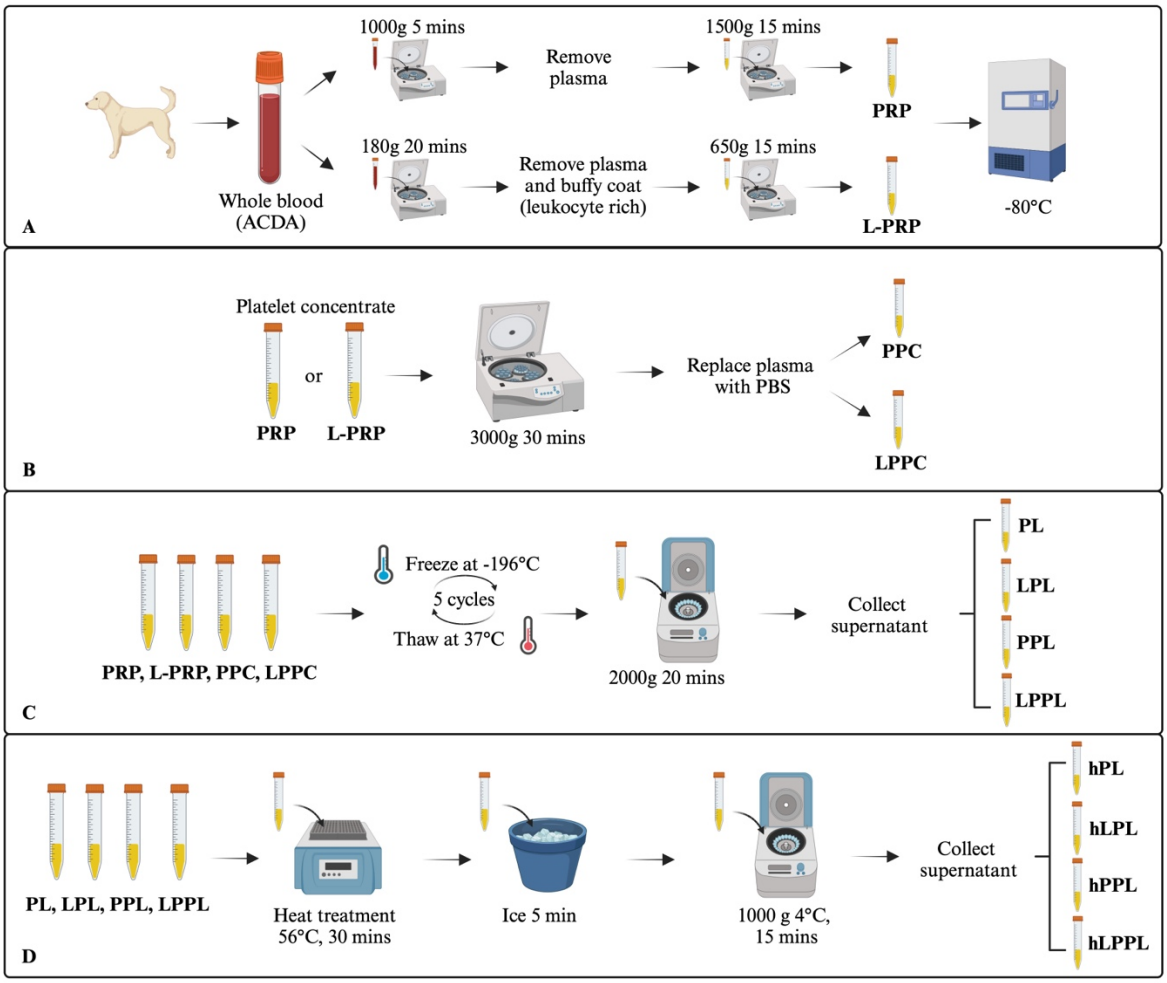

Figure 1: Overview of the study design. The image represents the experimental design and the procedures performed. (A) Generation of pure platelet concentrate (PC) and leukocyte rich platelet (LPC). (B) Plasma depletion and generation of pure platelet pellet concentrate (PPC) and leukocyte rich platelet pellet concentrate (LPPC). (C) Generation of lysate from PC, LPC, PPC and LPPC (PL, LPL, PPL, LPPL). (D) Heat treatment to generate complement inactivated samples from PL, LPL, PPL and LPPL (hPL, hLPL, hPPL and hLPPL). ACD-A, acid citrate dextrose-A solution; PC, pure platelet concentrate; LPC, leukocyte rich platelet concentrate; PPC; pure platelet pellet concentrate; LPPC; leukocyte rich platelet pellet concentrate; PL,

pure platelet lysate; LPL, leukocyte rich platelet lysate; PPL, pure platelet pellet lysate; LPPL, leukocyte rich platelet pellet lysate; hPL, heat-treated pure platelet lysate; hLPL, heat-treated leukocyte rich platelet lysate; hPPL, heat-treated pure platelet pellet lysate; hLPPL, heat-treated leukocyte rich platelet pellet lysate.

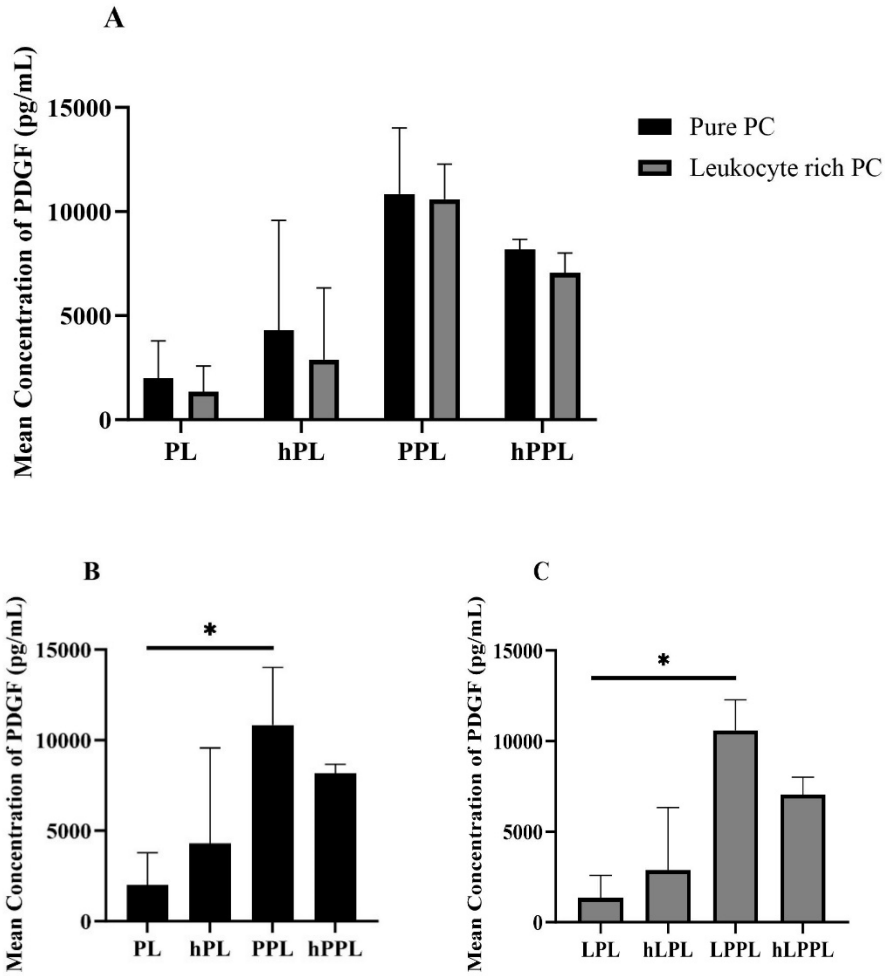

Figure 2: Mean concentration of platelet-derived growth factor (PDGF) between different preparations methods of lysate from both pure platelet concentrate (PC) or leukocyte rich platelet concentrate (LPC) (A), between lysates generated from PC (B), or LPC (C) through ELISA (n=2; mean ± SD). * $p < 0.05$. ELISA, enzyme-linked immunosorbent assay; PL, pure platelet lysate; LPL, leukocyte rich platelet lysate; PPL, platelet pellet lysate; LPPL, leukocyte rich platelet pellet lysate; hPL, heat-treated pure platelet lysate; hLPL, heat-treated leukocyte rich platelet lysate; hPPL, heat-treated pure platelet pellet lysate; hLPPL, heat-treated leukocyte rich platelet pellet lysate.

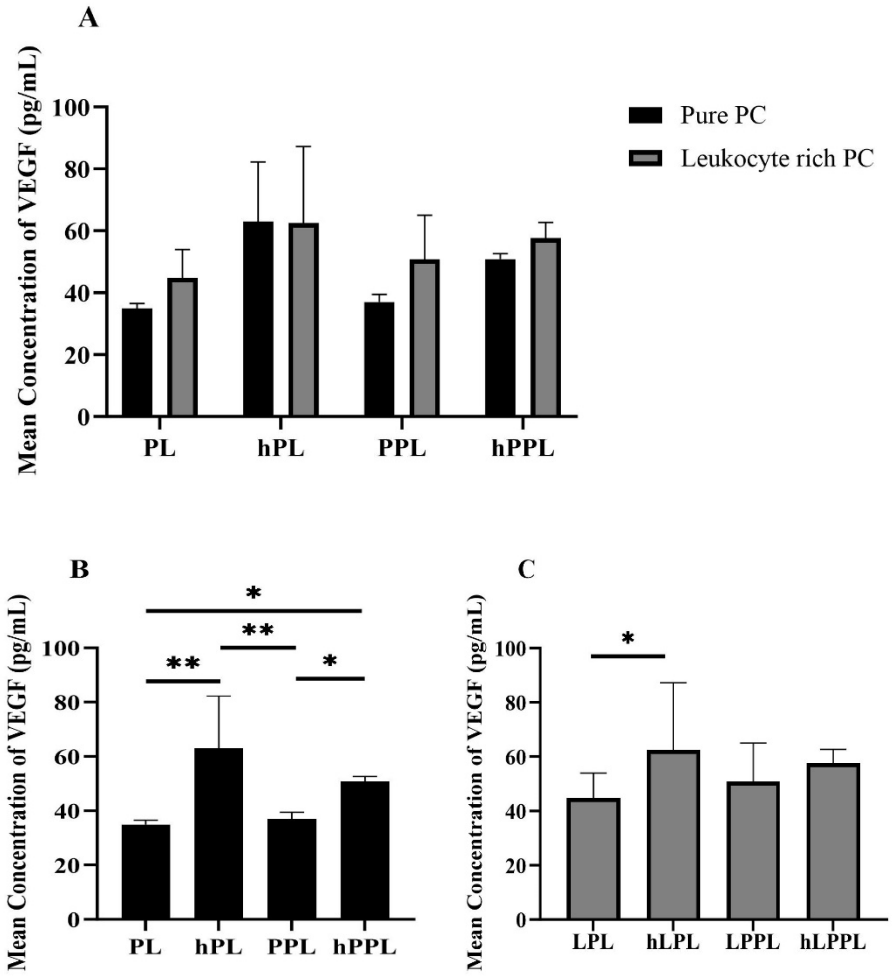

Figure 3. Quantification of vascular endothelial growth (VEGF) between different preparations methods of lysate from both pure platelet concentrate (PC) or leukocyte rich platelet concentrate (LPC) (A), between lysates generated from PC (B), or LPC (C) through ELISA (n=2; mean ± SD). * $p < 0.05$. ** $p < 0.01$. ELISA, enzyme-linked immunosorbent assay; PL, pure platelet lysate; LPL, leukocyte rich platelet lysate; PPL, platelet pellet lysate; LPPL, leukocyte rich platelet pellet lysate; hPL, heat-treated pure platelet lysate; hLPL, heat-treated leukocyte rich platelet lysate; hPPL, heat-treated pure platelet pellet lysate; hLPPL, heat-treated leukocyte rich platelet pellet lysate.

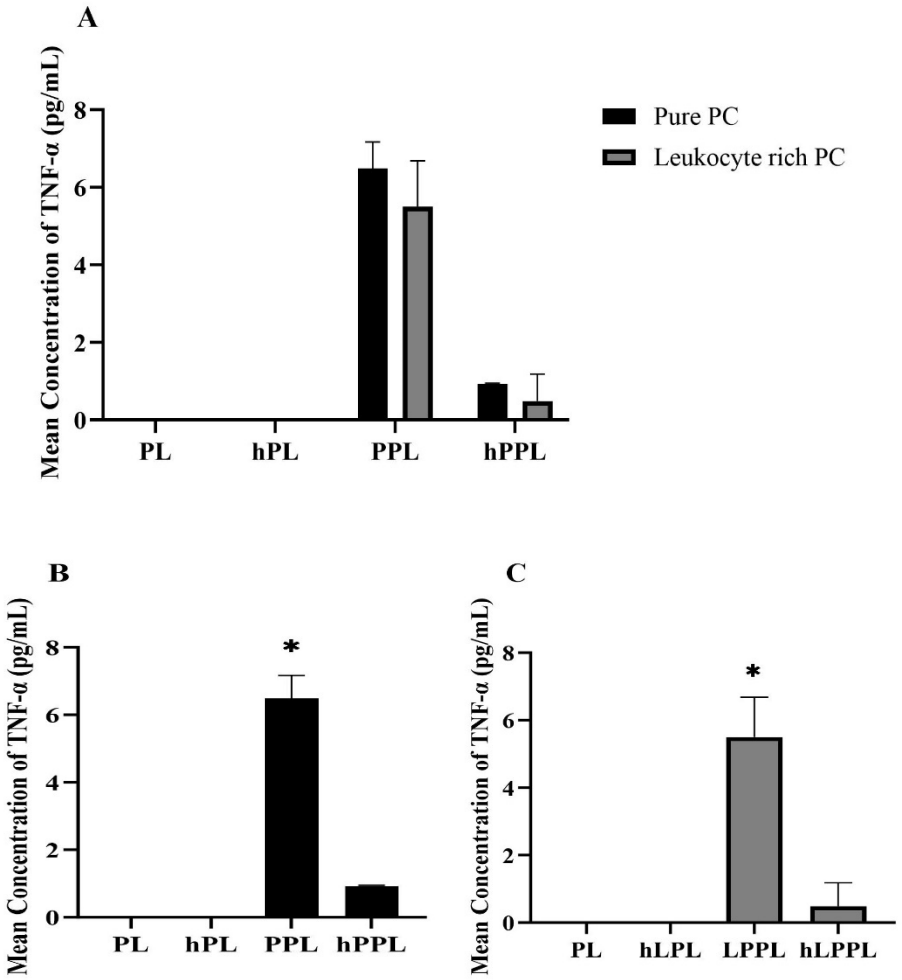

Figure 4. Quantification of tumor necrosis factor alpha (TNF-α) between different preparations methods of lysate from both pure platelet concentrate (PC) or leukocyte rich platelet concentrate (LPC) (A), between lysates generated from PC (B), or LPC (C) through ELISA (n=2; mean ± SD). * $p < 0.05$ compared to the rest of the groups. ELISA, enzyme-linked immunosorbent assay; PL, pure platelet lysate; LPL, leukocyte rich platelet lysate; PPL, platelet pellet lysate; LPPL, leukocyte rich platelet pellet lysate; hPL, heat-treated pure platelet lysate; hLPL, heat-treated leukocyte rich platelet lysate; hPPL, heat-treated pure platelet pellet lysate; hLPPL, heat-treated leukocyte rich platelet pellet lysate.

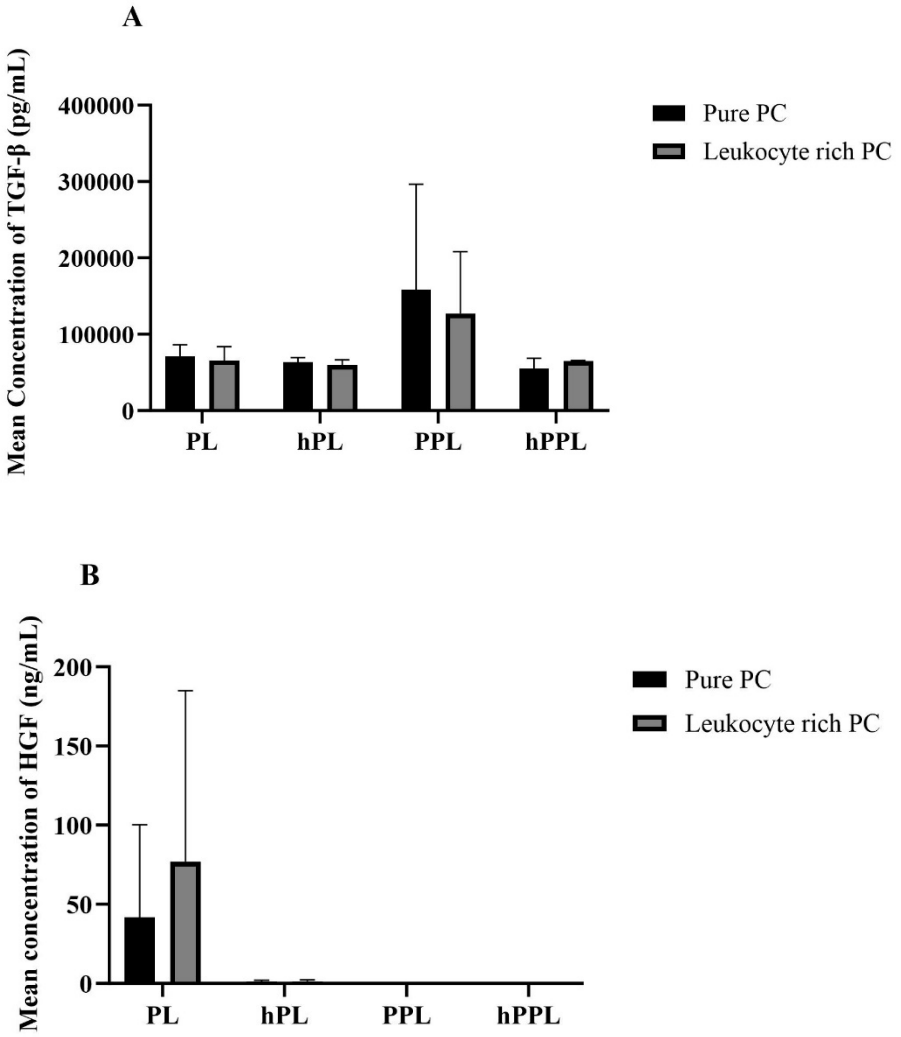

Figure 5. Quantification of transforming growth factor-β (TGF-β) (A) and hepatocyte growth factor (HGF) (B) from both pure platelet concentrate (PC) or leukocyte rich platelet concentrate (LPC) through ELISA (n=2; mean ± SD). ELISA, enzyme-linked immunosorbent assay; PL, pure platelet lysate; LPL, leukocyte rich platelet lysate; PPL, platelet pellet lysate; LPPL, leukocyte rich platelet pellet lysate; hPL, heat-treated pure platelet lysate; hLPL, heat-treated leukocyte rich platelet lysate; hPPL, heat-treated pure platelet pellet lysate; hLPPL, heat-treated leukocyte rich platelet pellet lysate.

CHAPTER 3

THE EFFECT OF VARIOUS PLATELET LYSATE FORMULATIONS ON THE VIABILITY, CHEMOTAXIS AND MIGRATION OF CANINE KERATINOCYTES OVER A PERIOD OF TIME[2]

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Introduction

Canine wounds can often be treated with primary or delayed primary closure [1]. However, patient and wound factors can lead to delayed wound healing which increases patient morbidity, wound infection risk, and cost to pet owners [2-4]. Managing chronic wounds remains a significant challenge because are difficult to heal, impair the animal's quality of life, and predispose to secondary complications, including necrosis and infection of deep tissues and bone [5]. Current therapies for wound management beyond primary closure or bandaging include negative pressure wound dressing, low-level laser therapy, photobiomodulation, tilapia and cod skin grafts, hyperbaric oxygen therapy, and bioelectric dressing [1, 3]. However, conventional treatments are ineffective in some cases [6], therefore, there is a need to develop alternative therapeutics that can accelerate healing and minimize associated complications.

Growth factors are important modulators of the physiological process of tissue repair by enhancing tissue regeneration through cell proliferation, extracellular matrix protein synthesis and degradation, angiogenesis, and chemotaxis [6, 7]. However, the administration of recombinant growth factors for wound healing is limited due to excessive cost and limited shelf availability for their routine clinical use [8, 9]. The enhanced comprehension of platelet function in wound healing led to the concept of using platelets as a natural source of growth factors as therapeutics tools [9]. Consequently, recent efforts have focused on developing platelet-derived products as an accessible and cost-effective source of growth factors, cytokines, chemokines, and proteins as a method to promote wound healing [7, 10-12].

Platelets play a pivotal role during the inflammatory phase of wound healing by releasing growth factors and cytokines responsible for the recruitment and activation of other

inflammatory cells, such as neutrophils and monocytes [13]. They also promote tissue regeneration, matrix remodeling, angiogenesis, and blood vessel maturation [9]. Specifically, the alpha granules within platelets contain numerous cytokines and growth factors that can affect tissue regeneration, reduce inflammation, and impact anabolic processes [14].

Platelet lysate is an acellular product rich in growth factors that can be stored for prolonged periods of time, while it can be used allogenically and be pooled from different donors to reduce individual donor-to-donor variability [15-17]. Topical application of platelet lysate has shown promising results for wound healing by promoting cell migration, keratinocyte epithelization, and regulating fibroblast matrix deposition [8, 18-20]. Additionally, recent evidence suggests that platelet-derived products hold analgesic and anti-inflammatory factors [21, 22] as well as antimicrobial properties in vitro and in vivo. These attributes make platelet lysate an appealing option for wound therapies.

There is great variability in the manufacturing of platelet-derived products. In our previous study, we found that variable leukocyte concentration and the presence of plasma proteins including heat-sensitive ones in the platelet lysate affect the release of growth factors and cytokines. Specifically, we found that the leukocyte rich platelet concentrate led to an increased concentration of growth factors important for wound healing. These factors can compromise the efficacy of the final product and impact the antimicrobial activity of platelet materials [23]. More specifically, plasma components such as fibrinogen and fibrin may also affect clinical outcomes as they can lead to persistent inflammation, causing scar tissue formation and ulceration [24]. In addition, fibrinogen can alter vasoconstriction, angiogenesis, and cell migration and proliferation [25]. On the other hand, the presence of fibrinogen and adhesive plasma proteins suspended in plasma may have advantageous effects on the healing

of both hard and soft tissue by the conversion into fibrin-based scaffold, which can initiate cell colonization and promote tissue regeneration [23]. Heat inactivation of complement can be implemented to deactivate proteolytic enzymes, remove unwanted proteins, and eliminate pro-thrombotic factors. This additional process prevents effects linked with toxicity caused by fibrin deposition [23, 24] and ensures biocompatibility and safety [24].

There are no previous studies directly evaluating how different preparation methods of canine platelet lysate affect *in vitro* cells associated with wound healing. Our objective was to assess how different concentrations of variable formulations of canine platelet lysate with or without plasma proteins and heat-sensitive proteins affect keratinocyte viability, chemotaxis, and migration *in vitro*.

Material and Methods

Blood collection

Six healthy canine blood donors (three male and three female beagles) aged 5.5-11.8 years old (median: 7.9 years), and body weights ranging from 13.5 - 38.8 kg (median: 19.40 kg) were included in this study that was approved by Auburn University Animal Care and Use Program (IACUC).

A total of 100 mL of whole blood was collected aseptically from the jugular vein using a 22 g needle from each donor and was divided into 8 mL blood collection tubes containing acid citrate dextrose-A solution (ACD-A; 3.2%). A small portion of blood was collected into an EDTA tube to perform a complete blood count (CBC) using ADVIA 2120 Hematology

Analyzer (Siemens Medical Solutions, Pennsylvania, USA) at the Clinical Pathology Laboratory at Auburn University immediately after the collection.

Generation of platelet concentrate

The platelet concentrate (PC) method was generated as described in Chapter 2 (growth factor and cytokine characterization of manual canine platelet lysate preparations with variable leukocyte concentrations, plasma content, and heat-sensitive proteins). Briefly, blood from each donor was divided into 15 mL colonial tubes and underwent a double-spin preparation. Blood was centrifuged at 180 g for 20 minutes. The plasma fraction and buffy coat layer were pooled from each tube and centrifuged at 650 g for 15 minutes to produce a platelet pellet. The plasma supernatant was carefully removed, and the platelet concentration was determined via CBC (Heska Element HT5, Heska, Colorado, USA). The platelet concentrates were resuspended with the appropriate amount of platelet poor plasma (PPP) to achieve a final concentration of 0.8 – 1 x 10^6/µL, as recommended by the American Red Cross for PRP [26].

Plasma depletion

For the plasma depletion, a portion of PC was centrifuged at 3000 g for 30 minutes. The plasma was removed and saved, and the platelet pellet was carefully resuspended with an equal volume of sterile phosphate-buffered saline (PBS) to produce plasma-free platelet pellet concentrate (PPC).

Generation of platelet lysate

Platelets were lysed via freeze/thaw cycles. PC and PPC were subjected to five freeze/thaw cycles in liquid nitrogen and thawing at 37 °C [27], and then centrifuged at 20,000 g for 20 minutes. This process produced platelet lysate (PL), and platelet pellet lysate (PPL).

Lysates within each group were pooled from three donors to generate two pooled batches to decrease individual variability.

Heat treatment for complement inactivation

A portion of pooled PL underwent heat treatment for complement inactivation in a dry bath at 56 °C for 30 minutes and immediately cooled on ice for at least 5 minutes. Then, centrifuged at 10,000 g for 15 minutes at 4 °C to remove any insoluble components as previously described [24]. This process produced heat-treated platelet lysate (hPL).

Culture of canine keratinocytes

Commercially available canine keratinocytes (CPEK, CELLnTEC, Advanced Cell Systems AG, Bern, Switzerland) were purchased and first expanded in keratinocyte media consistent with CnT-09 canine epithelial proliferation medium (CELLnTEC, Advanced Cell Systems AG, Bern, Switzerland) as per the protocol recommended by the manufacturer.

For all the experiments performed the keratinocyte media consisted of CnT complete media (CELLnTEC, Advanced Cell Systems AG, Bern, Switzerland). The starvation media consisted of CnT basal media supplemented with 0.1% W/V bovine serum albumin and 1% antibiotic mixture. Supplemented media consisted of CnT complete media supplemented with 10% v/v or 20% v/v PL, PPL, hPL, or PPP. Heparin (3 IU/ml, final concentration) was added to all formulations of platelet lysates to prevent coagulation and clotting during the cell cultures [28].

Viability assay

Cell viability was evaluated using the CyQUANT® XTT viability assay (ThermoFisher Scientific, USA). Specifically, keratinocytes were seeded onto a 96-well plate at a density of 8 x 10^3 keratinocytes per well and maintained in 100 µl of complete medium

(CnT-09 medium) for 24 hours in a 37 °C humidified atmosphere with a 5% CO_2 incubator. Subsequently, the medium was carefully removed and replaced with 200 µl/well of supplemented medium (CnT-09 medium) containing the different concentrations (10% v/v or 20% v/v) of each lysate formulation (PL, PPL, hPL, and PPP). Complete medium (CnT-09 medium) alone was used as a control. Viability was evaluated at 24 and 48 hours following the addition of the treatments and per manufacturer instructions. Briefly, the XTT reagent/electron coupling reagent was prepared and added onto each well plate and incubated for 4 hours in a 37 °C humidified incubator. The absorbance readings were obtained at 450 nm and 600 nm wavelengths using a microplate absorbance reader (BioTek Synergy H1 multimode reader, Agilent, CA, USA). The viability rate was expressed in percentage of change and normalized to the complete medium [($Absorbance_{lysate}$ formulation/$Absorbance_{media}$)*100]. Each treatment was performed in duplicate.

Chemotaxis assay

Keratinocyte chemotaxis assay was performed using a 6.5 mm transwell system with 8.0 µm pore polycarbonate membrane inserts (Corning Costar, Cambridge, MA, USA). A total of 3.5 x 105 keratinocytes were seeded with 100 µl of keratinocyte starvation medium in the upper chamber of each well. The lower chamber was filled with 650 µl starvation medium containing the variable lysate formulations (10% v/v or 20% v/v of PL, PPL, hPL, and PPP). Wells that were filled with the starvation media alone served as controls.

Keratinocytes were allowed to transmigrate for 6 hours in a 37 °C humidified atmosphere with 5% CO2 as previously described [8]. By using a cotton swab, cells that were attached but not migrated were removed from the upper side of the membrane. The membranes were fixed in 3.7% paraformaldehyde phosphate buffered saline (Boston BioProducts, MA,

USA) for 10 minutes and washed twice with PBS. Subsequently, the membranes were stained with Triton (0.5%) and Crystal Violet and mounted on microscope slides using ProLong® Gold Antifade Reagent with DAPI (Invitrogen Life Technologies, USA) and coversliped. Cells were counted in four different fields per transwell membrane under an inverted wide field fluorescent microscope (EVOS FLoid Imaging System, Invitrogen, ThermoFisher Scientific, CA, USA) and were counted using Image J software (NCBI) [29]. The chemotactic rate was expressed in percentage and normalized compared to starvation media [(number of cells migrated/number of cells in media alone)*100]. Each treatment was performed in duplicate.

Scratch closure assay

Using a 24-well plate, 8 x 10^4 keratinocytes were seeded with 500 µl/well of complete media (CnT-09 medium) into the plates and cultured to confluence in a 37 °C humidified atmosphere with a 5% CO_2 incubator. After reaching confluency, keratinocytes were starved for 24 hours. In confluent cell monolayers, a scratch was created by scraping across the monolayer with using a 0.1-10 µL pipette tip. Following PBS washes, suspended cells were re-fed with 500 µl/well of starvation media containing each treatment group (10% v/v or 20% v/v of PL, PPL, hPL, or PPP). Wells with only starvation medium (no supplements) served as control.

Cell migration into the denuded area was evaluated at 0, 6, and 24 hours after scratching, using an inverted microscope (DMi1 Inverted Microscope, Leica Microsystems Inc, USA), and 4 digital images in different zones were captured per well. The scratch area was measured using Image J software (NCBI) [29]. For qualitative evaluation, scratch closure was determined as the difference between scratch width at time zero and each time point. Closure rate was expressed in the percentage of scratch closure compared to the initial area

and was calculated as follows [(At0 – At)/(Media – Media0)]*100 [9], where At0 indicated scratch area at time 0, and T is the correspondent scratch area at 6 or 24 hours. Each treatment was performed in duplicate.

Results

Hematology values

The mean (± SD) concentration of platelets (PLT), white blood cells (WBC), and hematocrit (HCT) obtained from the whole blood and each platelet concentrate are illustrated in Table 1. The whole blood mean PLT concentration was 394.3 ± 164.34 x 10^3/μL, and the WBC concentration was 8.27 ± 1.67 x 10^3/μL. The PC had a 6.7-fold increase in platelet concentration (2,661 ± 1,150.89 x 10^3/μL) and 1.9-fold times increase in WBC concentration (15.7 ± 17.65 x 10^3/μL) compared to whole blood. The HCT was almost negligible for the PC. Negligible concentrations of platelets, WBC and HCT were identified in all lysate preparations (PL, PPL, hPL, and PPP).

Viability assay

Keratinocytes viability was normalized to the complete medium (assumed as 100%) is depicted in Figure 6. At 24 hours following the addition of the variable lysate formulations, a statistically significant lower viability rate was found following the addition of 10% PPP (96.37 ± 0.03 %; p = 0.021) compared to the media (Figure 6A). A statistically significant difference was not identified for the rest of the groups. At 48 hours the addition of 20% hPL resulted in a significantly lower viability rate of keratinocytes (77.05 ± 9.85 %; p = 0.043) compared to media alone. Whereas the addition of 20% PL resulted in a significantly higher

viability rate (98.64 ± 2.70%) than 10% PPL (86.00 ± 2.38 %; p = 0.021; Figure 6B). A statistically significant difference was not identified between the rest of the groups at the same time point. When we compared the same formulation of lysate between both time points, we found a statistically significant difference between 10% or 20% of PL, PPL, hPL and PPP between 24 and 48 hours (significant differences not depicted in the graphs). These data suggest that the viability rate of keratinocytes does not appear to be affected by the presence of variable lysate formulations with the exception of PPP and heat inactivated lysate. This is an important finding to ensure that cytotoxicity does not occur in the presence of the variable lysate formulations.

Chemotaxis assay

Chemotaxis data are depicted in Figure 7. Following the addition of 10% PL the chemotactic effect of keratinocytes was significantly improved with a 6.6-fold increase (256.44 ± 27.65%) compared to when 10% PPP was added to the culture (38.74 ± 36.93 %; p = 0.0471; Figure 7A). Subsequently, the addition of 20% PL (408.54 ± 18.03%) resulted in a significantly higher chemotactic effect compared to media only (p = 0.0063), 20% PPL (408.54 ± 18.03%, p = 0.211; Figure 7B), and 20% PPP (62.75 ± 9.98%, p = 0.0495; Figure 7B). Specifically, 20% PL resulted in a 2.5-fold increase (408.54 ± 18.03%) compared to 20% PPL (165.95 ± 15.93) and a 6.5-fold increase compared to 20% PPP (62.75 ± 9.98%). Finally, the addition of 20% hPL resulted in a significantly higher chemotactic effect (311.26 ± 132.14%) compared to 20% PPP (62.75 ± 9.98%, p = 0.0231) which corresponded to a 5-fold increase. These data suggested that plasma depletion and heat inactivation of lysate might affect the number of chemotactic cells compared to PL. Finally, it appears there is a less profound number of chemotactic cells in the presence of plasma alone, indicating that platelet content plays a role

in the migration of keratinocytes. Figure 8 shows representative figures of the migration rate of canine keratinocytes following the addition of variable formulations of platelet lysate.

Scratch closure assay

Scratch closure rate at 6 and 24 hours following the addition of the variable lysate formulations are illustrated in Figures 9A and 9B. Figure 10 shows representative photomicrographs of the keratinocyte scratch assay exposed to starvation medium alone (control) and starvation medium supplemented with all the various formulations of lysate at 0, 6, and 24 hours.

At 6 hours there was a significantly greater percentage of scratch closure rate when 10% of PL (114.21 ± 16.52) was added to the keratinocytes compared to media alone ($p = 0.0002$). The addition of 20% PPP (223.79 ± 245.68) resulted in a significantly higher closure percentage closure compared to media alone ($p = 0.0002$), nevertheless, the addition of 10% PPP (64.77 ± 23.03) did not achieve such an effect. The remainder of the formulations at 10 or 20% resulted in a significantly lower percentage of closure (10% PPL = 43.66 ± 7.93; 10% hPL = 96.39 ± 49.43; 10% PPP = 64.77 ± 23.03; PL 20% = 21.54 ± 48.98; PPL 20% = 76.75 ± 23.30; hPL 20% = 78.50 ± 5.82) compared to media alone.

Scratch closure rate at 24 hours is illustrated in Figure 9B. All platelet lysate formulations at both concentrations achieved a significantly higher percentage of scratch closure compared to media alone. More specifically, a statistically significant higher closure rate was found when 10% PL (371.40 ± 96.74%; $p = <0.0001$), 10% PPL (157.49 ± 12.61%; $p = 0.0015$), 10% hPL (282.05 ± 4.40%; $p = <0.0001$), 10% PPP (340.85 ± 223.96%; $p = <0.0001$), 20% PL (192.39 ± 189.19%; $p = <0.0001$), 20% PPL (244.08 ± 143.65%; $p = <0.0001$), 20% hPL (223.79 ± 245.68%; $p = 0.0002$), and 20% PPP (417.20 ± 49.66%; p =

<0.0001) were added to the keratinocyte culture compared to media alone. No statistically significant difference was observed between the variable lysate formulations. However, we noted that the addition of 10% PL tended to achieve a higher percentage of closure rate compared to the rest of the formations except for 20% of PPP at 48 hours. Plasma depletion and heat inactivation at both percentages seemed to accelerate the closure rate even though a significant difference was not encountered.

Discussion

In this study, we were able to show that different formulations and concentrations of canine platelet lysate affect *in vitro* the wound healing of canine keratinocytes. In order to characterize and test our hypothesis, we focused on major activities of the regenerative process, such as cell viability, chemotaxis, and migration.

Wound healing is a dynamic multicellular process characterized by a pattern of various events to restore the tissue architecture including hemostasis, inflammation, proliferation, and dermal remodeling [2, 13, 30-33]. Skin healing relies on the recruitment and coordinated functions of different cell types involved in different stages of this process, such as keratinocytes, endothelial cells, fibroblasts, inflammatory cells [6, 9], macrophages, and platelets to renovate the integrity of the skin barrier [18]. The complex process is regulated by multiple cytokines and growth factors released from the wounded epithelium, guiding the healing process with both stimulatory and inhibitory roles in different phases of healing [8, 9].

Initially, it was important to first identify whether the variable formulations of lysate affect the viability of keratinocytes and whether issues of cytotoxicity occur. The cell viability

assay performed in this study indicated that the keratinocytes maintained viability for up to 48 hours and a cytotoxic effect did not occur. However, the viability rate of keratinocytes seemed to decrease in the presence of variable formulations at both 24 and 48 hours compared to the control even though a significant difference was not identified between most of the groups. The exception to that was the addition of 10% PPP at 24 hours and 20% heat inactivated lysate (hPL) that significantly decreased the viability rate of keratinocytes compared to the media alone. It is important to note that the media was supplemented with only 10% or 20% of the specific lysate formulation, and it is possible that a higher concentration might be needed to increase the viability rate of keratinocytes. Thus, future studies should evaluate higher concentrations of the variable lysate formulations and their effect on keratinocyte viability. Finally, as expected, the addition of variable formulation of lysates (10 or 20%) achieved higher proliferation of keratinocytes between 24 and 48 hours.

Subsequently, we assessed the chemotactic activity of keratinocytes in the presence of variable lysate formulations. We found that the addition of 10% of PL resulted in a higher chemotactic effect compared to 10% of PPP. Even though not significant, we observed that plasma depletion and heat inactivation of the product led to a less pronounced chemotactic effect. A similar effect was also noted following the addition of 20% of the variable lysate formulations. The above findings suggest plasma components alone (PPP) do not seem to have a chemoattractant effect on keratinocytes and that platelet content is essential for an improved chemotactic activity of keratinocytes. Finally, plasma depletion and complement inactivation seem to affect the ability of lysate to modulate chemotaxis. This finding was in accordance with the study performed by Bijl et al., [37] which evaluated the chemotactic effect of platelet concentrates on peripheral blood mononuclear cells (PMBC) and found a higher chemotactic

effect in platelet in plasma (PC-plasma) compared to only plasma and platelets alone. In addition, they also found a higher migration and proliferation of human dermal fibroblasts in PC-plasma compared to plasma and platelets alone.

Furthermore, we aimed to characterize the effect of the variable lysate formulations on the migration of keratinocytes. For this purpose, we utilized the scratch closure assay which has been recognized as an efficient method for evaluating cell migration that can effectively simulate the in vivo behavior [38]. The wound closure rate was noted to be significantly accelerated for most formulations compared to media alone at 6 hours and for all formulations at 24 hours. This supports a sustained effect of platelet lysate for 24 hours on wound healing. Additional research is needed to evaluate how long these effects may last. Moreover, a significant difference was not seen between the variable formulations of lysate. However, while not significant, the addition of 20% PPP at both 6 and 24 hours and 10% PL at 24 hours tended to elicit an increased closure rate compared to the rest of the groups. The depletion of plasma and heat inactivation seemed to affect the closure rate, even though a significant difference was not seen.

In previous studies in humans, a dose-related effect was observed for the scratch closure rate and chemotaxis assay on primary human epidermal keratinocytes (HEK) [8]. In addition, a dose-related effect was also demonstrated in assessing cell viability in human umbilical vein endothelial cells (HUVECs), monocyte chemotaxis, and dermal keratinocyte migration [9]. More specifically, Jafar et al., [8] revealed that lower percentages (5% and 10%) of human PL showed a higher stimulatory effect on HEK scratch closure rate compared to 15% or 20% PL. Furthermore, El Backly et al., [20] found that 5% PL exerted the highest effect on scratch closure rate at 6 and 24 hours, and higher concentrations (10% and 20%) resulted in a

delayed effect compared to lower PL concentrations. However, it is important to highlight that the PL had a higher platelet concentration than this study (0.8 – 1 x 10^6/µL vs. 1 x 10^7/µL). However, Barsotti et al., [9] showed a higher scratch closure rate on human dermal keratinocytes with the addition of 10% and 20% platelet lysate at 48 and 72 hours compared to 5% platelet lysate. In our study, the concentration of platelet lysate (10% and 20%) with different formulations (presence or absence of plasma and complement proteins) did not seem to have differing effects on the viability and migration of canine keratinocytes.

The limitations of this study include that only two pooled lots of different formulations of canine platelet lysate were evaluated. In addition, we used immortalized healthy keratinocytes, and the effect of the lysate might be different when evaluated in primary cell cultures. Future studies are needed to evaluate how different lysate formulations affect other cell types important for wound healing, including fibroblasts, and immune cells such as neutrophils and macrophages. It is also important to evaluate the effect of platelet lysate on primary cell cultures of fibroblasts and keratinocytes or utilizing an ex vivo wound healing model. Lastly, chronic wounds can be complicated by secondary bacterial infection, evaluation of the antimicrobial activity of different formulations of platelet lysate may be beneficial.

Conclusions

In summary, our results proved that the formulation of platelet lysate affects *in vitro* the viability, chemotaxis, and migration of canine keratinocytes. Specifically, we found that plasma and platelet content are important for an improved chemotactic and migration activity

of canine keratinocytes. This is the first study proving the potential of canine platelet lysate to be used as an alternative therapy for wound healing.

References

1. Balsa, I.M. and W.T. Culp, Wound Care. Vet Clin North Am Small Anim Pract, 2015. 45(5): p. 1049-65.

2. Crovetti, G., et al., Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci, 2004. 30(2): p. 145-51.

3. Buote, N.J., Updates in Wound Management and Dressings. Vet Clin North Am Small Anim Pract, 2022. 52(2): p. 289-315.

4. Maciel, F.B., et al., Scanning electron microscopy and microbiological evaluation of equine burn wound repair after platelet-rich plasma gel treatment. Burns, 2012. 38(7): p. 1058-65.

5. Tambella, A.M., et al., Autologous platelet gel to treat chronic decubital ulcers: a randomized, blind controlled clinical trial in dogs. Vet Surg, 2014. 43(6): p. 726-33.

6. Ranzato, E., M. Patrone, L. Mazzucco, and B. Burlando, Platelet lysate stimulates wound repair of HaCaT keratinocytes. Br J Dermatol, 2008. 159(3): p. 537-45.

7. Shin, H.S., H.M. Woo, and B.J. Kang, Optimisation of a double-centrifugation method for preparation of canine platelet-rich plasma. BMC Vet Res, 2017. 13(1): p. 198.

8. Jafar, H., et al., Platelet lysate promotes the healing of long-standing diabetic foot ulcers: A report of two cases and in vitro study. Heliyon, 2020. 6(5): p. e03929.

9. Barsotti, M.C., et al., Effect of platelet lysate on human cells involved in different phases of wound healing. PLoS One, 2013. 8(12): p. e84753.

10. Gordon, J., S. Alvarez-Narvaez, and J.F. Peroni, Antimicrobial Effects of Equine Platelet Lysate. Front Vet Sci, 2021. 8: p. 703414.

11. Perazzi, A., et al., Description of a double centrifugation tube method for concentrating canine platelets. BMC Vet Res, 2013. 9: p. 146.

12. Kaushik, A. and M.S. Kumaran, Platelet-Rich Plasma: The Journey so Far ! Indian Dermatol Online J, 2020. 11(5): p. 685-692.

13. Barrientos, S., et al., Growth factors and cytokines in wound healing. Wound Repair Regen, 2008. 16(5): p. 585-601.

14. Arnoczky, S.P. and S. Sheibani-Rad, The basic science of platelet-rich plasma (PRP): what clinicians need to know. Sports Med Arthrosc Rev, 2013. 21(4): p. 180-5.

15. Hagen, A., et al., Platelet Lysate for Mesenchymal Stromal Cell Culture in the Canine and Equine Species: Analogous but Not the Same. Animals (Basel), 2022. 12(2).

16. Naskou, M.C., et al., Innate immune responses of equine monocytes cultured in equine platelet lysate. Vet Immunol Immunopathol, 2018. 195: p. 65-71.

17. Nebie, O., et al., The neuroprotective activity of heat-treated human platelet lysate biomaterials manufactured from outdated pathogen-reduced (amotosalen/UVA) platelet concentrates. J Biomed Sci, 2019. 26(1): p. 89.

18. Ranzato, E., et al., Scratch wound closure of C2C12 mouse myoblasts is enhanced by human platelet lysate. Cell Biol Int, 2009. 33(9): p. 911-7.

19. Ranzato, E., et al., Platelet lysate modulates MMP-2 and MMP-9 expression, matrix deposition and cell-to-matrix adhesion in keratinocytes and fibroblasts. Exp Dermatol, 2011. 20(4): p. 308-13.

20. El Backly, R., et al., Platelet lysate induces in vitro wound healing of human keratinocytes associated with a strong proinflammatory response. Tissue Eng Part A, 2011. 17(13-14): p. 1787-800.

21. Bendinelli, P., et al., Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF-kappaB inhibition via HGF. J Cell Physiol, 2010. 225(3): p. 757-66.

22. Mazzocca, A.D., et al., An in vitro evaluation of the anti-inflammatory effects of platelet-rich plasma, ketorolac, and methylprednisolone. Arthroscopy, 2013. 29(4): p. 675-83.

23. Delila, L., et al., Extensive characterization of the composition and functional activities of five preparations of human platelet lysates for dedicated clinical uses. Platelets, 2021. 32(2): p. 259-272.

24. Widyaningrum, R., et al., A purified human platelet pellet lysate rich in neurotrophic factors and antioxidants repairs and protects corneal endothelial cells from oxidative stress. Biomed Pharmacother, 2021. 142: p. 112046.

25. Copland, I.B., et al., The effect of platelet lysate fibrinogen on the functionality of MSCs in immunotherapy. Biomaterials, 2013. 34(32): p. 7840-50.

26. Dohan Ehrenfest, D.M., L. Rasmusson, and T. Albrektsson, Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). Trends Biotechnol, 2009. 27(3): p. 158-67.

27. Strandberg, G., et al., Standardizing the freeze-thaw preparation of growth factors from platelet lysate. Transfusion, 2017. 57(4): p. 1058-1065.

28. Naskou, M.C., et al., Platelet lysate as a novel serum-free media supplement for the culture of equine bone marrow-derived mesenchymal stem cells. Stem Cell Res Ther, 2018. 9(1): p. 75.

29. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis. Nat Methods, 2012. 9(7): p. 671-5.

30. Pinto, J.M., N.S. Pizani, H.C. Kang, and L.A. Silva, Application of platelet-rich plasma in the treatment of chronic skin ulcer - case report. An Bras Dermatol, 2014. 89(4): p. 638-40.

31. Iacopetti, I., et al., Autologous Platelet-Rich Plasma Enhances the Healing of Large Cutaneous Wounds in Dogs. Front Vet Sci, 2020. 7: p. 575449.

32. Wilkinson, H.N. and M.J. Hardman, Wound healing: cellular mechanisms and pathological outcomes. Open Biol, 2020. 10(9): p. 200223.

33. Theoret, C.L., The pathophysiology of wound repair. Vet Clin North Am Equine Pract, 2005. 21(1): p. 1-13.

34. Opneja, A., S. Kapoor, and E.X. Stavrou, Contribution of platelets, the coagulation and fibrinolytic systems to cutaneous wound healing. Thromb Res, 2019. 179: p. 56-63.

35. Bennett, N.T. and G.S. Schultz, Growth factors and wound healing: Part II. Role in normal and chronic wound healing. Am J Surg, 1993. 166(1): p. 74-81.

36. Gohel, M.S., et al., The relationship between cytokine concentrations and wound healing in chronic venous ulceration. J Vasc Surg, 2008. 48(5): p. 1272-7.

37. van der Bijl, I., M. Vlig, E. Middelkoop, and D. de Korte, Allogeneic platelet-rich plasma (PRP) is superior to platelets or plasma alone in stimulating fibroblast proliferation and migration, angiogenesis, and chemotaxis as relevant processes for wound healing. Transfusion, 2019. 59(11): p. 3492-3500.

38. Liang, C.C., A.Y. Park, and J.L. Guan, In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc, 2007. 2(2): p. 329-33.

Table 3: Descriptive results of hematological data presented as mean ± standard deviation from whole blood, and different platelet delivered concentrates. PLT, platelet concentration; WBC, white blood cell concentration; HTC, hematocrit; PC, platelet concentrate; PPC, platelet pellet concentrate; PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat treated PL.

	Hematology analysis		
PREPARATION	**PLT (x10^3/µL)**	**WBC (x10^3/µL)**	**HCT (%)**
Whole blood	394.3 ± 164.34	8.27 ± 1.67	51.33 ± 3.73
	Platelet concentrates		
PC	2,661 ± 1150.89	15.7 ± 17.65	1.80 ± 2.87
PPC	158.0 ± 74.97	0.09 ± 0.16	0 ± 0
PL	12 ± 1.41	0.01 ± 0	0 ± 0
PPL	3.50 ± 3.54	0.01 ± 0	0 ± 0
hPL	8 ± 7.07	0.06 ± 0.08	0 ± 0

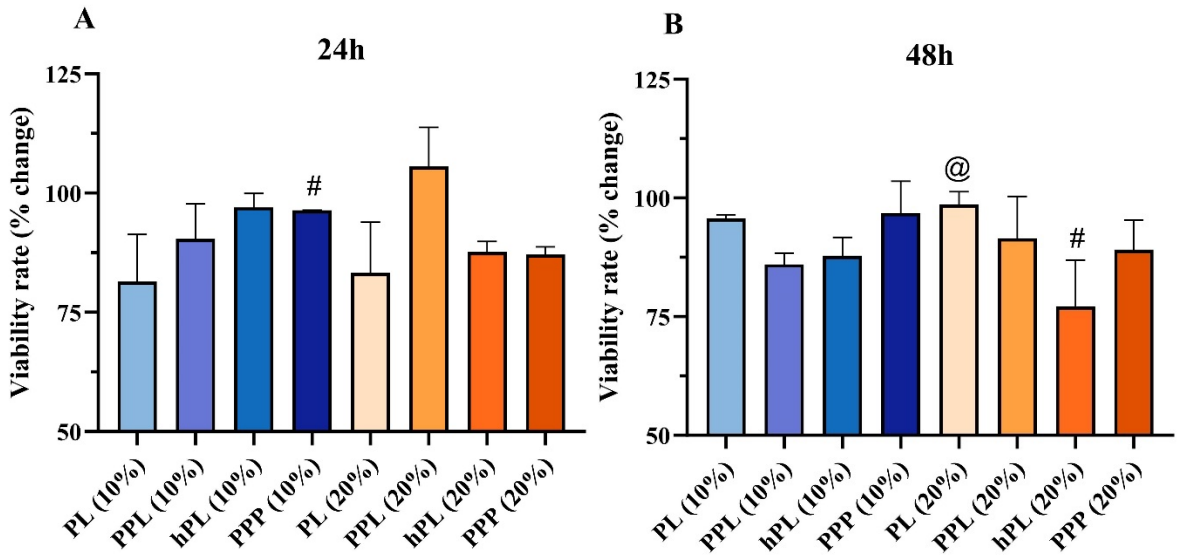

Figure 6: Viability of keratinocytes following the addition of variable formulations of platelet lysate. Keratinocytes viability was assessed by a colorimetric assay following the addition of 10 or 20% of the variable lysate formulations at 24 (A) and 48 hours (B). The viability rate was expressed in percentage of change and normalized to the complete medium [(Absorbance lysate formulation/Absorbance media)*100]. # significantly different to media; @ significantly different compared to 10% PPL. Data (n = 2) are mean ± SD of biological replicates. PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat-treated platelet lysate; PPP, platelet poor plasma.

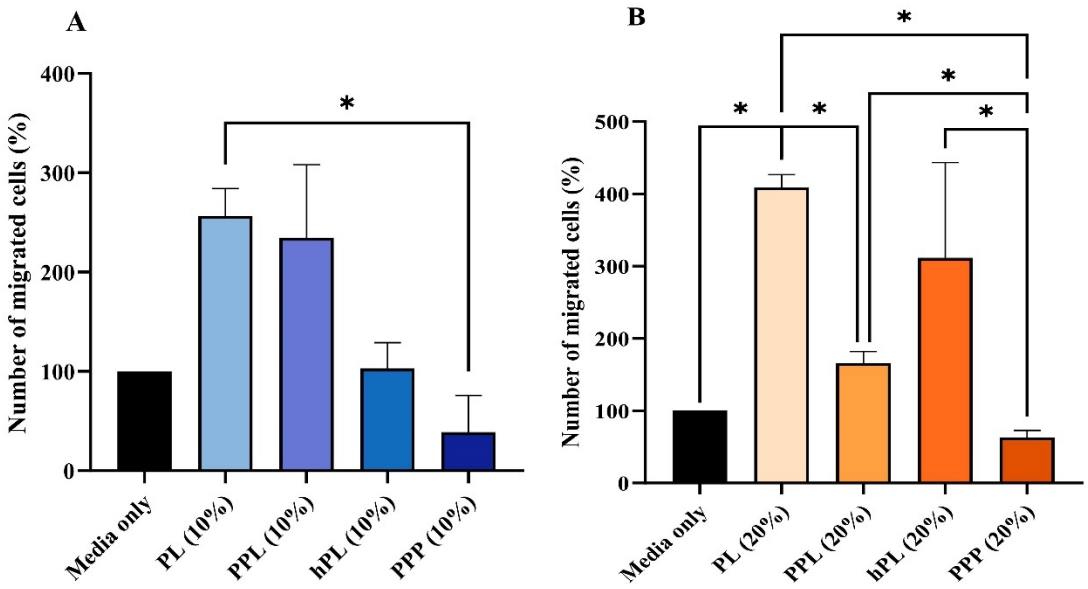

Figure 7: Chemotaxis of keratinocytes following the addition of variable formulations of platelet lysate. Effect of the addition of 10% (A) or 20% (B) of variable platelet lysate formulations on canine keratinocyte chemotaxis at 6 hours post addition. The chemotactic rate was expressed in percentage and normalized compared to starvation media and was calculated as follows [(number of cells migrated/number of cells in media alone)*100]. Data (n = 2) are mean ± SD of biological replicates. PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat-treated platelet lysate; PPP, platelet poor plasma.

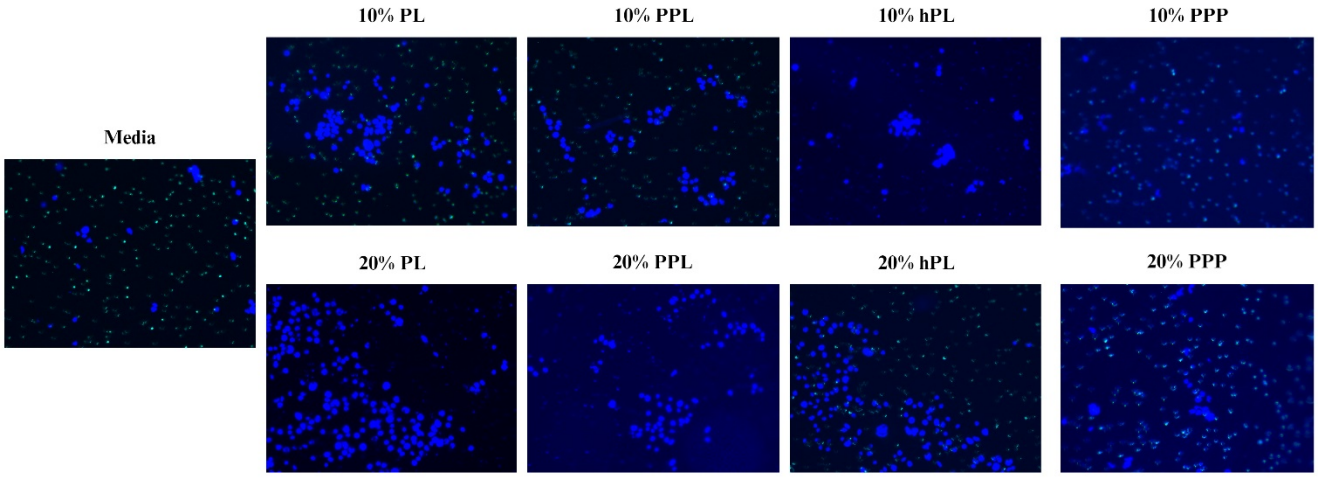

Figure 8: Immunofluorescence images of the migration rate of canine keratinocytes following the addition of variable formulations of platelet lysate. PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat-treated platelet lysate; PPP, platelet poor plasma.

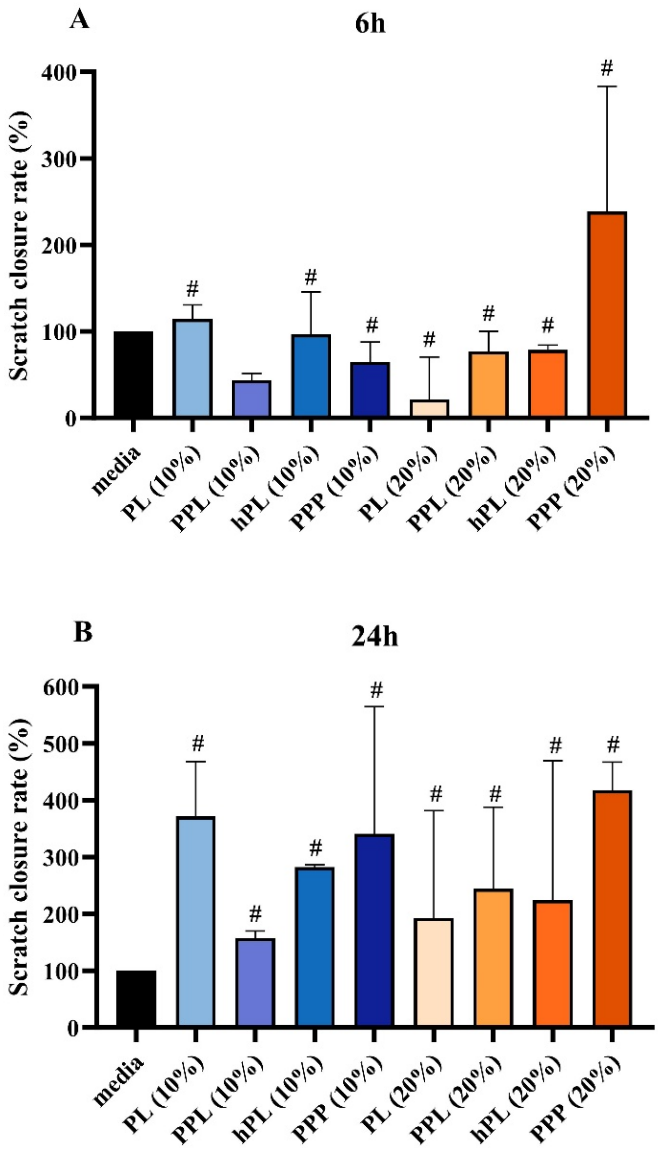

Figure 9: Scratch closure rate of keratinocytes following the addition of 10 or 20% of variable lysate at 6 hours (A) or 24 hours (B) post-scratch. Closure rate was expressed in the percentage of scratch closure compared to the initial area and was calculated as follows [(At0 – At)/(Media – Media0)]*100. # compared to media. Data (n = 2) are mean ± SD of biological replicates. PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat-treated platelet lysate; PPP, platelet poor plasma.

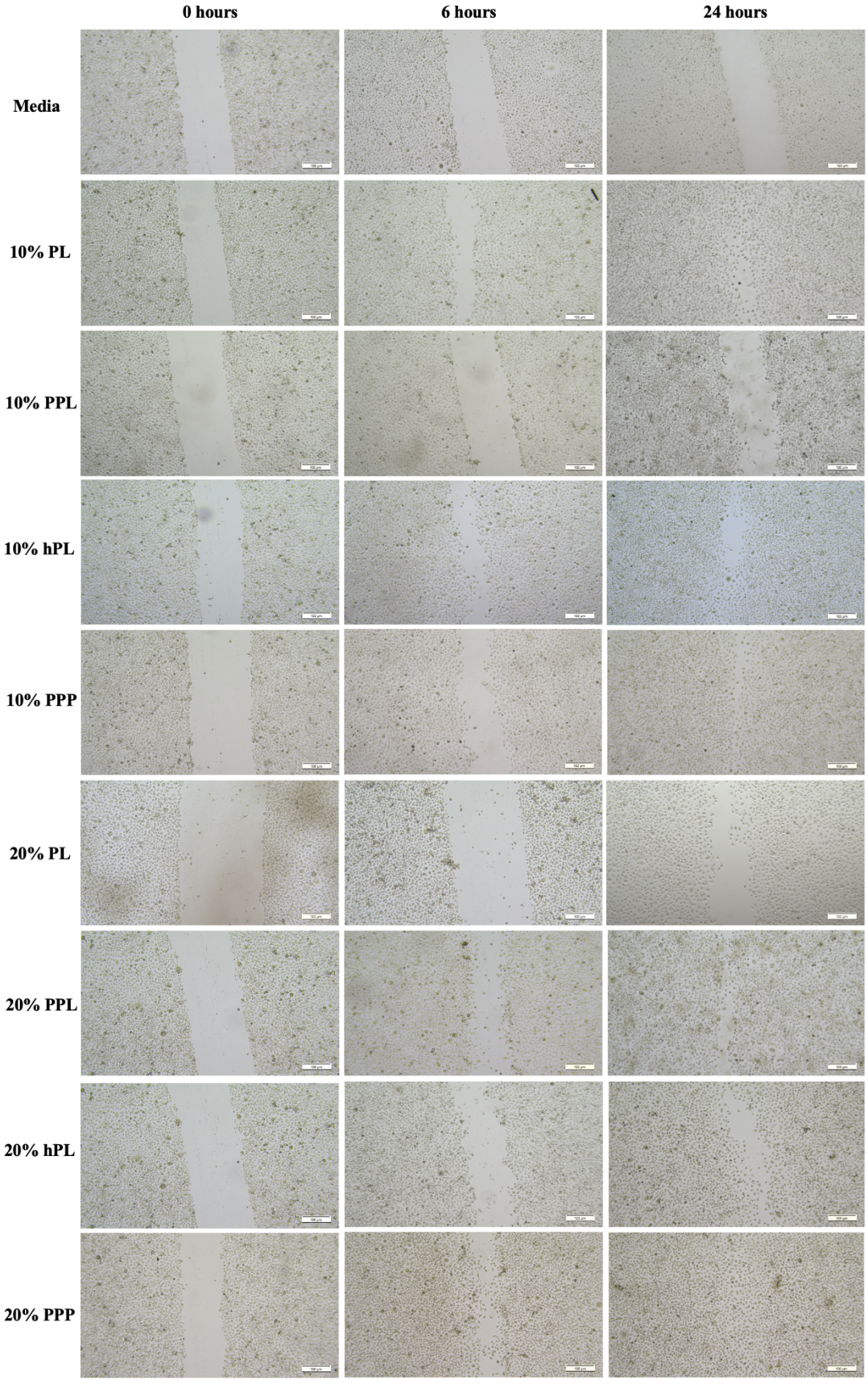
0 hours
6 hours
24 hours
Media
10% PL
10% PPL
10% hPL
10% PPP
20% PL
20% PPL
20% hPL
20% PPP

Figure 10: Representative phase contrast micrographs of the closure rate when keratinocytes were treated with 10% and 20% of the variable lysate formulations at 0, 6, and 24 hours. Scale bar: 100 μm. PL, platelet lysate; PPL, platelet pellet lysate; hPL, heat-treated platelet lysate; PPP, platelet poor plasma.

Conclusions

Millions of canine patients worldwide suffer from acute and chronic wounds resulting from chemical or physical trauma, bites, or burns with the vast majority recovering effectively. However, many factors can cause delays in healing, such as patient related conditions, and concurrent bacterial infections. Managing chronic wounds remains a significant challenge because the lesions are difficult to heal, impair the animal's quality of life, predispose to secondary complications, and can be a financial burden for the pet owners. Currently, canine wound management includes, among others, the application of topical and/or systemic antimicrobial agents and conventional antiseptic solutions. However, conventional treatments can be ineffective partially due to their inability to provide a nourishing environment to the wound, highlighting the need for alternative therapeutics that can accelerate healing and minimize associated complications.

Growth factors are important modulators of the physiological process of wound healing. However, the administration of recombinant growth factors is limited due to the high cost and limited shelf availability. Platelet-derived products, such as platelet lysate, have been proposed as an alternative therapy, offering an economical method to deliver a cocktail of growth factors that can promote wound healing.

Evidence suggests that variability in the manufacturing process of platelet-derived products and along with variations in their cellular composition, growth factor and inflammatory cytokine concentration, as well as the presence of specific plasma derived proteins, affect tissue metabolism both in vitro and in vivo and can compromise the efficacy of the final product. Chapter 2 of this thesis evaluated how different centrifugation methods

used to prepare platelet-derived products with variable leukocyte concentration, presence or absence of plasma proteins, and heat-sensitive proteins (complement) can affect the final concentration of growth and cytokine factors. The results of this study demonstrated that the formulation of platelet lysate and the presence of the leukocytes in the final product can influence the release of growth factors and cytokines with the potential to significantly impact the physiological and clinical efficacy of platelet-derived products.

Chapter 3 investigated the effects of different concentrations of various formulations of canine platelet lysate on the *in vitro* viability, chemotaxis, and migration rate of canine keratinocytes over a period of time. The results demonstrated that the composition of the platelet lysate significantly influences the major cellular activities of the healing process. We observed that the presence of plasma and platelet content are essential for an improved chemotactic and migration activity of canine keratinocytes.

The limitations of these studies include that the overall mean concentration of leukocytes between the two methods used to prepare a pure and leukocyte rich product did not strongly differ. Future studies should focus on the production of platelet-derived products with negligible or higher leukocyte concentrations. Furthermore, we assessed two lots of platelet lysate pooled from six canines, and future studies should include data from more biological replicates. For the investigation of the effect of lysate formulations on keratinocytes, we used commercially available canine keratinocytes from a healthy donor. It is possible that the effect of the lysate might be different when evaluated in primary cell cultures from wounded dogs. Future studies are needed to evaluate how various lysate formulations affect other cell types important for wound healing, including fibroblasts and immune cells such as neutrophils and macrophages. Additionally, before clinical application it is important to evaluate the effect of

platelet lysate on using an ex vivo wound healing model. Finally, given that chronic wounds are often complicated by secondary bacterial infection, evaluating the antimicrobial activity of different formulations of canine platelet lysate is important.