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USE OF AUTOLOGOUS PLATELET CONCENTRATES FOR THE TREATMENT OF MUSCULOSKELETAL INJURIES IN THE HORSE

Preliminary clinical studies and cellular and molecular evaluation of equine platelet concentrates obtained by single and double centrifugation tube methods

PhD Thesis

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Academic dissertation approved for the partial fulfillment of the requirements for the degree of doctor of philosophy by the Universitat Autònoma de Barcelona, Spain

This work is dedicated to my mother, my family, and my wife

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LIST OF COMMON ABBREVIATIONS

Actin binding protein	ABP
Beta thromboglobulin	β-TG
Cross sectional area	CSA
Cyclo-oxigenase-2	COX-2
Degree of lameness	DL
Deep digital flexor tendon	DDFT
Desmitis of the branches of the suspensory ligament	BDSL
Desmopathy of the suspensory ligament	DSL
Epidermal growth factor	EGF
Epithelial neutrophil-activating protein-78	ENA-78
Equine recombinant insulin like growth factor type-1	erIGF-I
Extracellular Matrix	ECM
Fibroblast growth factor	FGF
Glycoprotein	GP
Glycosaminoglicans	GAGs
Growth factors	GFs
Growth-regulating oncogene alpha	GRO-α
Hepatocyte growth factor	HGF
Hyaluronic acid	HA
Insulin like growth factor	IGF
Interferon gamma	IFN-γ
Interleukin 1	IL-1
Intracellular cell adhesion molecule type 1	ICAM-1
IL-1 receptor antagonist	IL-1ra
Joint effusion	JE
Large unstained cells	LUCs
Leukocytes (white blood cells)	WBCs
Leukotriene B4	LTB_4
Lipopolysaccharides	LPS
Macrophage inflammatory protein 1 alpha	MIP1-α
Matrix metalloprotease degrading enzymes	MMPs
Maximum injury zone	MIZ

Mean platelet component concentration	MPC
Mean platelet component distribution width	MPCDW
Mean platelet volume	MPV
Megakaryocyte	MK
Mesenchymal stromal cells	MSCs
Monocyte chemotactic protein-3	MCP-3
Nitric oxide	NO
Non-steroidal anti-inflammatory drugs	NSAIDs
Nuclear factor kappa-beta	NF-κB
Osteoarthritis	OA
Osteochondrosis	OCD
Packed cellular volume	PCV
Platelet	PLT
Platelet activating factor	PAF
Platelet-endothelial cell adhesion molecule	PECAM
Platelet concentrate	PC
Platelet Factor-4	PF-4
Platelet derived growth factor	PDGF
Platelet distribution width	PDW
Platelet rich plasma	PRP
Prostaglandin E_2	PGE_2
Proximal desmopathy of the suspensory ligament	PDSL
Red blood cells	RBCs
Regulated on activation normal T expressed and secreted	RANTES
Superficial digital flexor tendon	SDFT
Suspensory ligament	SL
Synovial fluid	SF
Tisular inhibitors of MMPs	TIMPs
Transforming growth factor beta	TGF-β
Type II collagen	col-II
Tumor necrosis factor alpha	TNF-α
Vascular cell adhesion molecule type 1	VCAM-1
Vascular endothelial growth factor	VEGF
von Willebrand factor	vWF

GENERAL SUMMARY

Platelets (PLTs) play a central role in wound healing, since they contain growth factors (GFs), which produce chemotaxis, cellular proliferation and differentiation, neovascularization, and extra-cellular matrix (ECM) deposition. The use of autologous platelet concentrates (PCs) has been proposed to accelerate wound repair and to stimulate the regenerative capacity of injured tissues. Two clinical studies about the effect of an autologous platelet concentrate (PC) in horses with severe musculoskeletal pathology are presented in chapters III and IV of this thesis. The PC was prepared by a novel double centrifugation tube method. A cellular and molecular characterization of this PC is presented in chapter V of this thesis

The effect of the intraarticular injection of this PC in 7 horses with severe joint disease was evaluated on the basis on degree of lameness (DL) and joint effusion (JE). When PCs were injected into the joint a statistically significant improvement in both DL and JE ($p<0.05$) were observed. The most marked improvement was maximun 2 months after the last injection and persisted up to 8 months later (*see* chapter III). The clinical effect of PC injection in 7 horses with soft tissue musculoskeletal injuries namely: SDFT tendinopathy and desmopathy of the susensory ligament (DSL) was also evaluated (*see* the chapter IV). All the horses presented a clinical and a statistical ($p<0.05$) decrease of the DL and the response to flexion test. Ultrasonic appearance improved in the horses with SDFT lesions, but remained the same in the horses with DSL. Two horses with acute SDFT tendinopathy returned successfully to competition level without reinjury. One horse with chronic SDFT tendinopathy relapsed. The rest of the horses with DSL returned successfully to competition level without reinjury. A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg TGF-β_1 were obtained per ml of the PC. No adverse reactions resulted from this treatment.

A cellular and a molecular study of the PC clinically used in this thesis (PC-C),of whole blood and of three additional PCs (PC-A, PC-B, and PC-D) obtained during the PC-C preparation were performed (*see* the chapter V) to compare the single and the double centrifugation tube methods for concentrating equine platelets. Whole blood and the 4 PCs were analyzed using flow cytometry for cellular quantification and determination of TGF-β_1 in all the samples. Platelet concentrations for PC-A, PC-B, PC-C and PC-D were 45%, 44%, 71% and 21% higher, respectively, compared to the same values for citrated whole blood samples. TGF-β_1 concentrations for PC-A, PC-B, PC-C and PC-D were 38%, 44%, 44% and 37% higher, respectively, compared to citrated whole blood sample values. In conclusion, the single and double centrifugation tube

methods are reliable methods for concentrating equine platelets and obtaining potentially therapeutic TGF-β_1 levels.

The results obtained in this thesis open a new encouraging research field on clinical and molecular effects of PCs in equine chronic musculoskeletal pathology. The future potential results obtained in horses can be of key value to determine the potenetial use of autologous PCs in human beings with chronic musculoskeletal pathology.

RESUMEN GENERAL

Las plaquetas (PLTs) son protagonistas en la reparación de las heridas, ya que contienen factores de crecimiento (GFs), los cuales producen quimiotaxis, proliferación y diferenciación celular, neovascularización y deposición de matriz extracelular (ECM). Se ha propuesto la utilización de concentrados de plaquetas (PCs) autólogos para acelerar la reparación de las heridas y estimular la capacidad de regeneración de los tejidos lesionados. En los capitulos III y IV de esta tesis se presentan dos estudios clínicos sobre el efecto de un concentrado plaquetario (PC) autólogo en caballos con patología músculo-esquelética grave. Este PC fue preparado por medio de una nueva técnica de doble centrifugación en tubo. Una caracterización celular y molecular de este PC se presenta en el cápitulo V de ésta tesis.

Se evaluó el efecto de la inyección intra-articular del PC en 7 caballos con enfermedad articular grave clasificada así según el grado de cojera (DL) y la efusión sinovial (JE). El PC produjo una mejoría estadísticamente significativa del DL y JE ($p<0.05$). La mejoría más notable fue observada a los dos meses de finalizado el tratamiento y permaneció hasta 8 meses después (*ver* cápitulo III). En el capitulo IV se evaluó el efecto del PC en 7 caballos con lesiones tendinosas (tendonitis del tendón flexor digital superficial (SDFT) y ligamentosas (desmitis del ligamento suspensorio (DSL)). Todos los caballos mejoraron significativamente su DL y la respuesta a la prueba de flexión ($p<0.05$). Los registros ultrasonográficos mejoraron en los caballos con tendinopatías, pero no cambiaron en los que tenían desmopatías. Dos caballos con tendinitis retornaron con exito a su nivel de competición sin recidivar. Un caballo con tendinosis crónica recidivó. El resto de pacientes con desmopatías volvieron a su nivel de competición pre-lesión. Se obtuvieron un promedio de 250 ± 71.8 x 10^6 plaquetas, 8.68 ± 3.78 leucocitos x 10^6 y 12515 ± 2443 pg de TGF-β_1/ml de PC. No se observaron signos clínicos adversos asociados con el tratamiento.

En el cápitulo V se realizó un análisis celular y molecular del PC usado clínicamente (PC-C), sangre entera y 4 fracciones de PCs adicionales (PC-A, PC-B y PC-D), los cuales son obtenidos durante la elaboración del PC-C. El objetivo fue evaluar el método de obtención de este PC, en el que son necesarios dos periodos de centrifugación. Todas las muestras fueron analizadas mediante citometría de flujo y determinación de los niveles de TGF-β_1. Las concentraciones de plaquetas para PC-A, PC-B, PC-C y PC-D fueron un 45%, 44%, 71% y 21%, respectivamente más altas en comparación con cada PC y la sangre entera. Las concentraciones de TGF-β_1 para PC-A, PC-B, PC-C y PC-D fueron un 38%, 44%, 44% and 37%, respectivamente más altas en comparación con cada PC y la sangre entera. Se concluyó que el método empleado para concentrar plaquetas es valido para producir PCs con niveles potencialmente terapéuticos de TGF-β_1.

Los resultados obtenidos en esta tesis abren un nuevo y prometedor campo de investigación para conocer los efectos clínicos y moleculares de los PCs en caballos con enfermedad crónica músculo-esquelética. Los resultados que se puedan obtener en caballos serán de gran valor para conocer el uso potencial de los PCs autólogos en seres humanos con similares patologías.

I. GENERAL INTRODUCTION

GENERAL INTRODUCTION

Motivation

Lameness due to musculoskeletal pathology is a frequent problem in equine athletes. In horses, as in people, most musculoskeletal diseases are related to chronic degenerative processes of one or several locomotor structures, such as joints, tendons, and ligaments (1, 2). Osteoarthritis (OA) (1, 4), superficial digital flexor tendon (SDFT) tendinopathy (5) and suspensory ligament (SL) desmopathy (6) are the most documented chronic musculoskeletal pathologies of the horse (7, 8). OA is a degenerative process of the articular cartilage and is considered the most important joint disease in equine practice (2-4). This pathology has a similar pathophysiologic process in humans (1, 9), horses (2-4) and dogs (10). Degenerative tendinopathies of the SDFT and the deep digital flexor tendon (DDFT) are frequent in horses (5-8). In human beings other tendons are more frequently affected (mainly, the Achilles tendon and the shoulder rotator cuff, amongst others) (11), but the pathophysiologic mechanism of the all these processes seems to be similar (11-17). Ligaments can also be affected by degenerative processes and in the horse, this problem is of a paramount importance (6). The specialized anatomic conformation of the distal region of the equine limb predisposes to the development of desmitis of the SL (18).

Articular cartilage, tendons and ligaments are specialized connective tissues. Microscopically, these tissues are formed by resident cells imbibed in an extracellular matrix (ECM). Articular cartilage is formed by chondrocytes surrounded by an ECM which contains type II collagen and non-collagenous proteins, such as glycosaminoglicans (GAGs) (chondroitin sulfate, keratan sulfate and aggrecan), and hyaluronic acid (HA), amongst others (1, 2, 4, 9, 10). Tendons and ligaments are composed by specialized fibroblasts, named tenocytes and myofibroblasts, which are imbibed in a type I collagen and other GAGs ECM (11-13). The molecular and cellular behavior of these specialized connective tissues has been extensively documented during the past two decades. To date, it is recognized that resident cells of these tissues are active and they participate in the normal ECM turnover process by producing numerous cellular mediators, such as cytokines, growth factors (GFs), and metalloprotease degrading enzymes (MMPs), amongst others (2, 4, 16).

The balance between anabolism and catabolism of the ECM and the viability of the resident cells is of pivotal importance for maintaining the health of the specialized connective tissues, such as cartilage, tendon, and ligament. Many factors, including ageing, excessive or inadequate exercise protocols and inappropriate rest could increase the metabolic demand of these structures and resident cells could either die (apoptosis) (19-21) or fail to produce enough quality ECM (1, 2, 4).

The result of these inadequate processes is a weakened tissue, which does not respond to the animal's locomotor demands and is more prone to re-injury (3, 22-25).

The molecular and microstructural knowledge of the pathophysiology of chronic degenerative musculoskeletal pathologies in man, horses and other animals has opened new encouraging issues for the treatment of these pathologies. Very important pathologic events have been observed during chronic degenerative musculoskeletal pathologies. An exaggerated expression of catabolic cytokines (mainly, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α)) (1, 2, 9, 16) up-regulate the production of MMPs, eicosanoids (prostaglandin E2 (PGE_2) leukotriene B4 (LTB_4)), and free radicals (specially, nitric oxide (NO) (4, 9, 26, 27). Finally, this molecular inflammatory process is accompanied by the death (aberrant apoptosis) of resident cells (19-21). Symptomatic treatment of these pathologies with classical drugs such as corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) is becoming outdated and can even be detrimental for patients with chronic degenerative musculoskeletal diseases (28).

Recently, novel therapeutic approaches, such as the use of recombinant GFs (29-43), gene-therapy (44, 45), or mesenchymal stromal cells (MSCs) (46-48), amongst others, have been suggested for the treatment of OA, tendinopathies and desmopathies in man, horses and other animals. Most of these novel treatments have been experimentally used or assessed in phase I or II human clinical trials. In horses, the use of equine recombinant insulin like growth factor type-1 (erIGF-I) for the treatment of SDFT tendinopathy has been experimentally evaluated and the results of this research seem encouraging. The clinical use of this GF has been recommended for the treatment of clinical cases of equine SDFT tendinopathies (33). However, this treatment is expensive and there is still not enough scientific data to support its positive effects for the treatment of clinical cases.

Recently, McIlwraith´s research team at Colorado State University evaluated the effects of gene-therapy for the treatment of experimentally induced equine OA (44). They infected the synovium of osteoarthritic horses with an adenovirus which carried the IL-1 receptor antagonist (IL-1ra) encoding gene. The results were positive; however the effects of this treatment lasted only 1 month and horses developed moderate synovitis (44). Up to date, the use of gene-therapy for the treatment of horses with clinical musculoskeletal pathologies has not been reported.

The use of MSCs or autologous bone marrow injection has been proposed for the treatment of horses with a SDFT tendinopathy (48) and SL desmopathy (46). The results of these studies are promising; however, autologous purified MSCs were used only in one horse (48). It is known that bone marrow contains MSCs and GFs, which can have a positive effect in horses with soft tissue musculoskeletal pathologies (48). However, the use of bone marrow is not exempt of side effects,

such a heterotopic calcification post-injection or the introduction of bone spicules (46). In this sense, the use of autologous purified expanded MSCs for the treatment of musculoskeletal chronic injuries in horses could be a more suitable (46) option, although this treatment is expensive and there is a lack of information about its clinical use and side sffects.

Some points should be considered regarding the use of novel treatments in horses and even in humans: cost-efficacy, adverse immunologic reactions, and the availability of advanced technology. It is important to bear in mind that recombinant GFs are very expensive, consecutive doses are required, and its positive effects have not been clinically tested in a high number of cases (4). The use of MSCs has the same restrictions than GFs, although it is hypothesized that these cells produce regenerative effects and possibly their repeated use is not necessary (46-48). Gene-therapy seems to be encouraging. However, many problems related with to the biology of the viral vectors used in its development, the transient expression of the targeted genes, and the adverse immunologic reactions, amongst others, need to be solved before clinical use is recomended(44, 45).

The platelet α-granules contain a variety of GFs, which can or cannot be specific for platelets (PLTs). The main GFs secreted by platelets include: transforming growth factor beta-1 (TGF-β_1), TGF-β_2, platelet derived growth factor AA (PDGF-AA), PDGF-BB, PDGF-AB, IGF-I, epidermal growth factor (EGF), and hepatocyte growth factor (HGF). Platelet concentrates (PCs) are an important source of autologous GFs (49). PCs have been used in human medicine for augmenting alveolar-maxillary reconstruction (49, 50), plastic (51) and orthopedic surgery (52). In addition, there are anecdotic reports about the use of PCs in people for the management of Achilles tendinopathy (52) and other soft tissue musculoskeletal chronic lesions, and for the treatment of severe arthropathies including OA and rheumatoid arthritis with encouraging results (R Soler et al. unpublished data). It has been suggested that the supraphysiological concentrations of GFs present in PCs could work in a positive way by accelerating wound healing, decreasing the inflammatory reaction, and promoting the regeneration rather than the repair of the affected tissues (49, 51).

The positive results observed in human patients affected by chronic musculoskeletal pathologies treated with PCs were a motivation to begin the doctoral thesis presented here. This document describes the use of PCs for the treatment of horses with severe joint disease (OA and osteochondrosis (OCD)) (*see* **Section III** of this thesis) and chronic soft tissue locomotor pathologies (SDFT tendinopathy and SL desmitis) (*see* **Section IV** of this thesis). PCs used in this study were obtained by a modified protocol (53) of the double centrifugation tube method developed in our facilities.

Hypothesis

Three general hypotheses (2 clinical and 1 basic) were evaluated in this thesis:

1. The intra-articular or perilesional injection of a PC obtained by the double centrifugation tube method can be useful for the treatment of equine patients with musculoskeletal pathologies, including severe arthropathies (OA and OCD), and soft tissue locomotor pathologies (SDFT tendinopathy and, SL desmopathy).
2. The use of PCs for the treatment of equine musculoskeletal pathologies is safe.
3. The number of PLTs, WBCs and the levels of TGF-β_1 should be different from whole blood and when comparing the four PC fractions (PC-A, PC-B, PC-D, PC-C) obtained during the preparation of the PC used clinically in this thesis.

General objectives

1. To evaluate the clinical and side effects of an autologous PC in horses with chronic musculoskeletal pathologies (*see* **Sections III** and **IV**).
2. To study the cellular and molecular levels of the PC used therapeutically in this study (PC-C), whole blood and three additional PCs (PC-A, PC-B, and PC-D), which are produced during the preparation of the PC-C (*see* **Section V**).
3. To validate the tube method technique for obtaining autologous PCs derived from equine blood.

Specific objectives

For **Sections III** and **IV:**

1. To evaluate the clinical effects of the intra-articular injection of the autologous PC, obtained with the double centrifugation tube method in horses affected with severe joint disease, through the assessment of the degree of lameness and joint effusion.
2. To evaluate the clinical effects of the peri-lesional injection of the PC obtained by double centrifugation tube method in horses affected with soft tissue musculoskeletal pathologies, by means of ultrasound image analysis and evaluation of the degree of lameness.
3. To establish a protocol of treatment with an autologous equine PC, and to report the possible side effects associated to this treatment.

For **Section V:**

1. To evaluate PLT count, WBC count and packed cell volume (PCV) in equine whole blood, PC-C and three additional PCs (PC-A, PC-B, and PC-D) obtained by the single and the double centrifugation tube methods using a flow cytometry hematology system.
2. To determine the TGF-β_1 levels with an ELISA technique in equine whole blood, PC-C and three additional PCs (PC-A, PC-B, and PC-D) obtained with the single and the double centrifugation tube methods.

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II. REVIEW OF THE LITERATURE

REVIEW OF THE LITERATURE

Summary

Platelets (PLTs) play a very important role in wound healing, since they secrete many growth factors (GFs) and other molecules involved in this process. The main GFs secreted by PLTs include: platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β_1), TGF-β_2, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin like growth factor type 1 (IGF-I) and hepatocyte growth factor (HGF). These peptides produce chemotaxis, cellular proliferation and differentiation, neovascularization, and extra-cellular matrix (ECM) deposition, which favor the resolution of inflammation and the healing of the wounds. The use of autologous platelet concentrates (PCs) has been proposed in order to accelerate wound repair and to decrease the inflammation related to trauma. It has also been proposed to stimulate the regenerative capacity of the tissues injured, avoiding the production of repair tissue and eventually an unfunctional scar. In human medicine, there is data that supports the positive effects of PCs in maxillary-alveolar, plastic and orthopedic surgery. Many procedures have been developed to concentrate the higlest number of platelets. To date, the optimal dose of PLTs that should be used in these surgical procedures remains unknown. Two methods for concentrating equine platelets have recently been validated, the apheresis and the buffy coat methods. However, there is a lack of knowledge about the clinical use of PCs in equine medicine. There is only one not well-controlled study that describes encouraging effects of PCs for the treatment of experimentally induced skin wounds in a horse.

Introduction

Wound healing is a fascinating process directed by a myriad of cellular and molecular mechanisms. Many cells are involved in wound healing and they produce or are sensible to many molecules (e.g.: cytokines, growth factors (GFs), and eicosanoids amongst others), which allow, in physiologic conditions, the repair or even the regeneration of injured tissues (1). Platelets (PLTs) play a central role in the wound healing process, since this cytoplasmic fragment possesses not only haemostatic properties (2), but also pro-inflammatory, regulatory (3), and regenerative activities mediated by the interaction with cells (namely neutrophils, and endothelial cells) and by liberating GFs, chemokines and other regulatory molecules (4).

Recently, the use of autologous platelet concentrates (PCs) for stimulating wound healing has increased in human oral-maxillofacial (4-7), plastic (8, 9) and orthopedic surgery(10, 11). The use of PCs in equine medicine seems to be encouraging for the treatment of limb wounds (12) and chronic musculoskeletal conditions. The general objective of this review is to describe how PCs can be potentially useful in the horse to improve the wound healing process in different tissues, including cartilage, tendon, ligament, bone and skin. Thus, a description about PLT genesis, physiology, biochemistry, pathobiology of wound healing, the methods used for preparing human and equine PCs, their clinical use and the potential sideeffects derived from autologous PC use are described in this review.

Platelet genesis, physiology, and biochemistry

Megakaryocytes (MKs) are the precursors of PLTs. These cells develop from multipotential progenitor CD34+ myeloid cells which reside in the hemopoietic tissue and the bloodstream. MKs represent approximately 0.1-0.5% of the nucleated marrow cells. MKs are located beneath the capillary sinuses in the marrow and emit cytoplasmic prolongations, proplatelets (Fig 2.1.a), which are in contact with the blood. These prolongations are sectioned and PLTs are released to bloodstream (Fig 2.1.b) (2, 13).

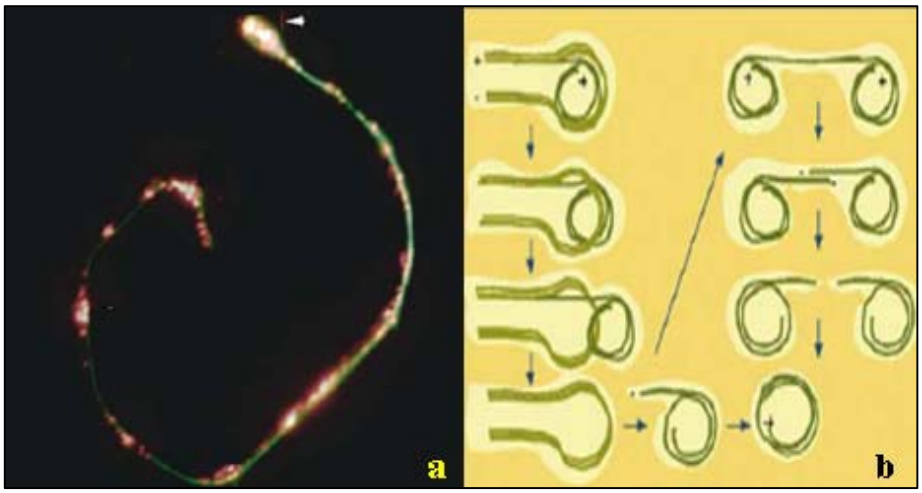

Figure 2.1. a) Electronic micro-photography of a megakaryocytic proplatelet. Note the relealse of a platelet at the end of proplatelet (*see blank arrow*) b) Schematic hypothetical representation of the platelet release from a megakaryocytic proplatelet. Modified from reference 2.

Megacaryocytic proliferation is a necessary step for PLT genesis. This phase is stimulated by several cytokines (e.g.: interleukin (IL)-3, IL-6, and IL-11) and thrombopoietin. Mature MKs suffer constant cytoplasmic expansion that is filled with cytoskeletal proteins (α and β tubulin, actin, myosin polymers, actin binding protein (ABP), gelosin, profilin, talin, vinculin and tropomyosin), specific granules, granular contents, and membranous systems (**Fig 2.2**). Although it is recognized that PLTs originate from cytoplasmic elongations of the megakaryocyte, the physiological mechanism by which PLTs are produced is not well understood (2, 13).

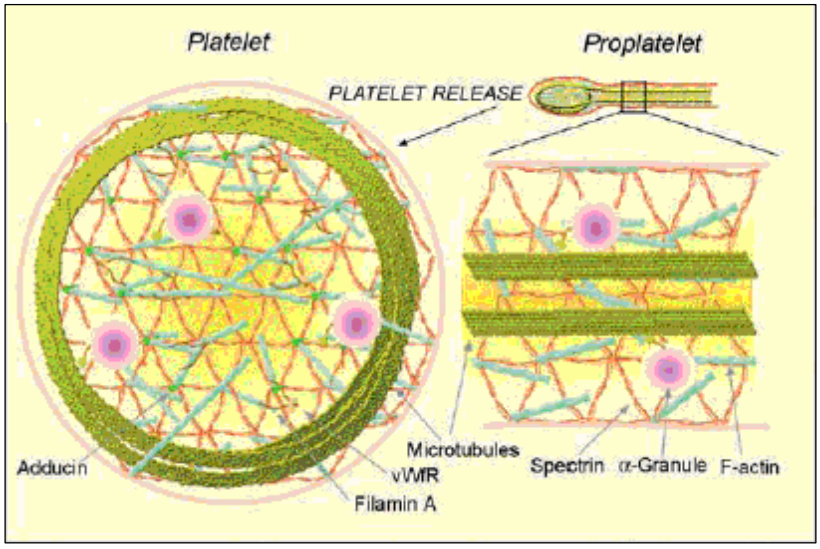

Figure 2.2. Schematic representation of the disposition of different platelet components that are released from the megakaryocytic proplatelet. vWfR: von Willebrand factor. Modified from reference 2.

The equine platelet is an anucleated discoid cytoplasmic fragment 5-7 μm long and 1-3 μm wide; although in some conditions large PLTs (>20 μm) are aften seen in the bloodstream (13). An electronic microphotography of a human activated platelet is shown in figure 2.3.

Platelet membrane

The platelet membrane has three layers the glycocalix, the phospholipidic layer, and the submembranous layer (14). The glycocalyx is the outer layer and contains glycoproteic receptors implicated in PLT activation and adhesion. These glycoproteins constitute the PLT membrane antigens, which are divided in three families: integrins, leucine-rich proteins and selectins. An asymmetric phospholipidic bilayer with anticoagulant properties constitutes the central layer the structure of this layer is identical to that of other cells with transmembranous and peripheral protein domains that act as membrane receptors. Phospholipids are layed out forming opposing rows with the polar projection inwards (14).

Negatively charged lipids, aminophospholipids (phosphatidylserine (PS), and phosphatidyl enthalonamine (PE) constitute the inner portion of the phospholipidic membrane. Neutral phospholipids (phosphatidilcholine (PCh) and sphingosine) are present in the outer sheet of the phospholipidic membrane. Other lipids present within the membrane include: phosphatidyl inositol (PI), and lysolecithin (15). The inner or submembranous layer is a part of the cytoskeleton and binds to some glycoproetins of the outer layer. (14)

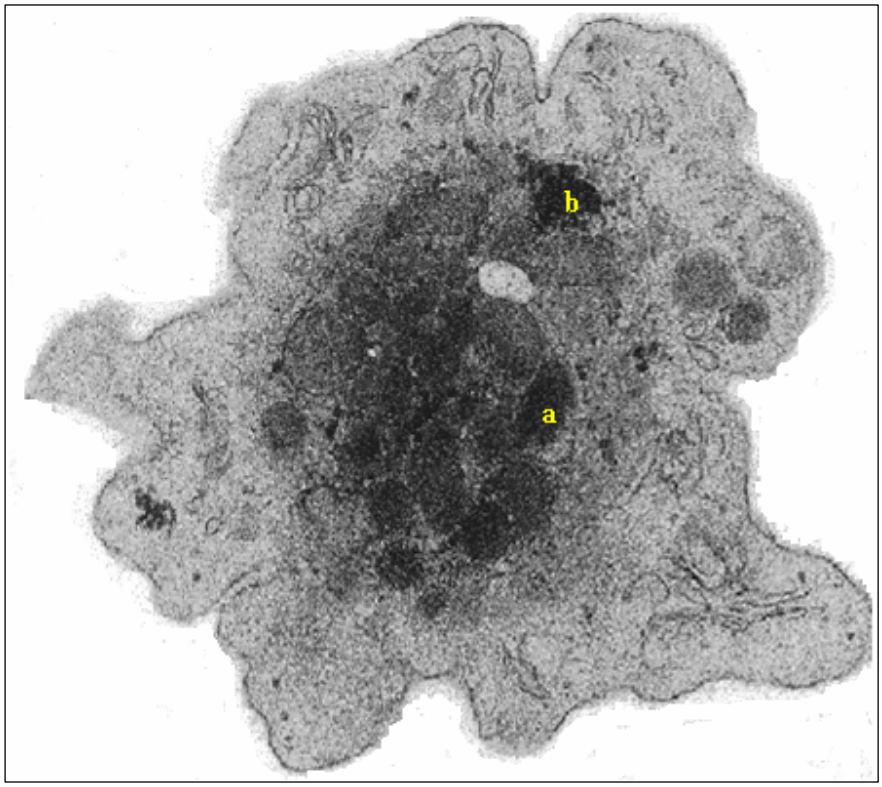

Figure 2.3. Electronic microphotography of an activated human platelet. Note the emission of cytoplasmic peudopodia **a)** Granular contents (α-granules, amongst others) are centralized before being released **b)** Glycogen vacuoles. (Modified from Anítua E. Un nuevo enfoque en la regeneración ósea. Plasma rico en factores de crecimiento (PRGF).Ed. Puesta al Dia Publicaciones. Vitoria. 2000).

Integrins

Integrins are proteins involved in a large number of cellular interactions. With PLTs, they produce aggregation and adhesion (14, 16, 17). The basic structure of integrins is constituted by two subunits, α and β, which are not covalently bound. Integrins are connected internally with the PLT cytoplasm by a single C-terminus tail and externally with the media by several subunits with extracellular domains (N-terminus). The inner (cytoplasmic tail) portion of integrins is associated with signaling proteins (G proteins, tyrosine kinases) and phosphoinositides. $Beta_1$ and β_3 are the most important integrin subunits foun in PLTs. PLT β_1 subunit is mainly associated with three

different α subunits. The β_3 subunit is related with the glycoprotein (GP) IIb-IIIa ($\alpha IIb\beta_3a$) and $\alpha v\beta_3$ (vitronectin receptor). GPIIb-IIIa, also know as P-selectin, is the main integrin of PLTs and MKs. This GP is present on the surface of α-granules and interacts with fibrinogen, the von Willebrand factor (vWF), fibronectin and vitronectin. The externalization of this GP is related with PLT activation (3, 4, 14, 16, 17).

Leucine-rich glycoproteins and other platelet receptors

Leucine-rich glycoproteins, mainly GPIb-IX-V, contribute to the net negative charge on the PLT surface. These glycoproteins have two subunits, GPIbα and GPIbβ. The outer domain contains receptors for vWF and thrombin. The inner cytoplasmic tail is associated with ABP. When vWF binds to these GPs there is an increase in cytoplasmic calcium and activation of phosphatidyl inositol-3-kinase. These phenomena produce the activation of phopholipase A_2 and the synthesis of eicosanois, namely thromboxane A_2. Other PLT membrane receptors include: thrombin receptor, immunoglobulin receptors (platelet-endothelial cell adhesion molecule (PECAM)-1), thrombospondin receptor and ADP receptor, amongst others (14, 16).

Platelet cytoplasm

PLT cytoplasm is constituted by the same cytoplasmic proteins than the megakaryocyte (2, 13). Two types of actin are present in PLTs, globular and filamentous, and they constitute the cytoplasmic network. Actin filaments act as a structural support for different PLT granules and mitochondria. PLT response is by a contractile activity mediated by actin-myosin polymerization. Cytoplasmic microtubules maintain the discoid form of PLTs and direct the movements generated by actin-myosin. The adequate assemblage of the PLT cytoskeleton is paramount for granule centralization, secretion and clot retraction (*see* **Fig 2.2**) (2, 14, 16).

Platelet granules

Mammal PLTs contain three types of granules: lysosomal, dense and alpha (α) granules (*see* **Fig 2.3**) (2-4, 14, 16, 18). Lysosomal granules contain acid hydrolases, guanine, phospholypases and kinases, which act as proteolytic and hydrolytic enzymes (14). Dense granules store ATP, ADP, calcium, phosphorus and serotonin. ADP induces PLT migration and in combination with serotonin produces contraction of injured arteries. ATP antagonizes ADP action (18). Alpha granules contain several molecules (cytokines, chemokines, growth factors, amongst others); some of them are specific for PLTs (e.g.: PF-4 and β-thromboglobulin (β-TG)) and others such as albumin, proteoglycans (chondroitin 4-sulphate), fibrinogen, fibronectin, thrombospondin, factor V, factor Va and von Willebrand factor which are not specific for PLTs (2-4). These proteins are important for all the platelet functions, including: thrombus formation and growth, inflammatory modulation and synthesis of ECM during wound healing, amongst others (16). The α-granules store

mainly seven GFs directly implicated in wound healing, including: platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β_1), TGF-β_2, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin like growth factor type 1 (IGF-I) and hepatocyte growth factor (HGF) (4, 5).

Platelet derived growth factor

The PDGF family consists of PDGF-A, -B, -C, and –D. These peptides can be found as homodimeric or heterodimeric forms (PDGF-AA, -BB, -AB, -CC, and DD). The four PDGFs are inactive in their monomeric forms. Two PDGF receptors (PDGFRs) have been identified, α and β, that dimerize when dimeric PDGF binds. Expression of both receptors and each of the four PDGFs is under an independent control that allows for a high degree of combinatorial flexibility. In addition, α_2- macroglobulin binds in a reversible fashion with PDGF, forming a complex which serves to avoid binding of PDGF with its receptors, protects PDGF from proteolytic degradation and removes PDGF from circulation via α_2- macroglobulin receptors (19).

PDGFs play a paramount role during embriogenesis and in many physiologic and pathologic mechanisms of adult animals (19). In wound healing, PDGF is a powerful chemoattractant and stimulator of cell proliferation. PDGF stimulates angiogenesis and up-regulates the expression of MMP-1 and its natural inhibitor (TIMP-1) in the late phase of remodeling. PDGF produces fibroblast proliferation, epithelial migration, extensive vascularization and neutrophil infiltration. PDGF is highly regulated by TGF-β during the normal wound healing process (1, 19, 20).

Transforming growth factor beta

TGF-β_1, TGF-β_2, TGF-β_3, and numerous bone morphogenetic proteins (BMPs), belong to the TGF-β superfamily (21). TGF-βs are produced by macrophages, lymphocytes, endothelial cells, fibroblasts, astrocytes, osteoblasts, osteoclasts and PLTs (22). TGF-β_1 and TGF-β_2 are the most representative GFs contained in PLTs (4). TGF-βs are secreted as latent precursor peptides (LTGF-β) which require activation into a mature form for receptor binding and subsequent activation of signal transduction pathways (23). LTGF-β can be activated either by proteases such as plasmin and thrombin or by physical interactions with proteins like thrombospondin-1 or integrins. Nitric oxide (NO) also activates TGF-β (23). Cellular actions of active TGF-β are mediated by five TGF-β receptors (TβRs), although TβRI and TβRII are the most implicated in normal functions of this peptide (21).

TGF-β is a pleiotropic molecule that may stimulate or inhibit cell proliferation, differentiation, motility, adhesion or death, depending on the type and the developmental state of a cell (1, 22). Proliferation of epithelial, endothelial, hematopoietic, neural and mesenchymal cells is down-regulated by TGF-β (21). In addition, this peptide has an important activity on ECM synthesis (22), since it regulates the expression of fibrilar collagens and fibronecticn (24). TGF-β also represses ECM degradation by down-regulating MMPs, promoting the synthesis of their natural inhibitors (TIMPs) and of fibroblast-derived growth factor, and producing angiogenesis (22). It is important to point out that TGF-β is paratiount in the regulation of the immunological system, since it regulates multiple stages of T cell apoptosis, selection, activation and clearance (21).

TGF-β is vital for wound healing and its balanced expression is necessary for the formation of an optimal and cosmetic scar, since when TGF-β (especially TGF-β_1) is pathologically up-regulated, a hiperthrophied or cheloid scar ensues (1, 22). On the other hand, TGF-β_3 has antifibrotic properties and is up regulated during fetal development.Wen it binds to TβRI, TGF-β_1 produces cell proliferation; when bound to TβRII it induces ECM synthesis (25). Equine and human TGF-β_1 share an aminoacidic sequential homology of 99% (26).

Epidermal growth factor

EGF consists of a single chain polypeptide produced by epithelial cells, fibroblasts and PLTs. This peptide and TGF-α bind to a single specific receptor (EGFR), which can trigger processes like wound healing, central nervous system development, craniofacial morphogenesis and even cancer (20). In wound healing, EGF can induce cell proliferation, differentiation and motility. EGF is highly expressed in the margin of wounds promoting re-epithelization (27). MMP-1 facilitates epithelial cell migration across the collagen type I matrix in several tissues, including the dermis. EGF up-regulates MMP-1 expression and regulates type I collagen turnover (20).The sequence of EGF has been determined in the horse and presents a 60-70% homology with human EGF (28).

Vascular endothelial growth factor

VEGF belongs to the VEGF gene family, which includes placenta growth factor, VEGF-B, -C, -D and two VEGF like proteins. VEGF, also known as VEGF-A, has four predominant isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Endothelial vascular cells and PLTs produce

VEGF. VEGF-A produces its biological effects when it binds to two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (29). VEGF has proliferative effects on vascular endothelial cells with an additional pro-survival effect for these cells (20, 25). VEGF also promotes the proliferation of retinal pigment epithelial, pancreatic duct and Schwann cells *in vitro* (20). VEGF is a powerful angiogenic peptide implicated in embryogenesis, adult maintenance and carcinogenesis (29). VEGF-A is vital for endochondral bone formation and its down-regulation is related to developmental skeletal growth defects (20). In wound healing, VEGF promotes the vascularization of injured tissues and thus facilitates the arrival of inflammatory and reparative cells (1, 20, 27). Up to now, the DNA sequence of equine EGF has not been reported.

Fibroblast growth factor

FGFs represent an extended family of polypeptides with a high affinity for heparin. FGFs act binding and activatig FGF tyrosine kinase receptors (FGFRs). FGF–FGFR interaction and signaling are further regulated by the spatial and temporal expression of endogenous heparan sulfate proteoglycan. At least seven isoforms of FGF (FGF-1, -2, -4, -7, -9, -10, and -19) have been identified; although FGF-1 (basic FGF (bFGF)) is the most studied peptide in wound healing (20, 25). Connective tissue cells and PLTs produce FGF-1, which induces proliferation of fibroblasts, keratinocytes, endotelial and smooth muscle cells. This peptide has powerful angiogenic properties and controls ECM deposition together with TGF-β, since bFGF down-regulates type I collagen synthesis (1, 25). In addition, bFGF promotes retinal regeneration (20).

Insulin-like growth factor

Insulin-like growth factors are two molecules (IGF-I and IGF II) from the insulin family that are produced by many tissues, specially the liver (30). Blood plasma is IGFs' main reservoir. IGF-I is captured by an endocytic mechanism before its storage in PLTs (4). This peptide is transported by 6 binding proteins (IGFBPs) which modulate their biological action (30). Young animal tissues express IGF-I in abundance, but its levels diminish in older animals (31). IGF-I is a single chain autocrine factor that binds to a specific cell surface receptor (IGF-I-R) and stimulates direct differentiation and ECM synthesis maintenance (20). IGF is a powerful anabolic peptide of pivotal importance in the wound healing process. This peptide shares a high homology between human and equine species (31).

Hepatocyte growth factor

HGF is a heparin-binding protein that is mitogenic for endothelial and epithelial cells (32). This peptide has powerful angiogenic effects, since it increases VEGF expression. HGF and VEGF show a synergistic action on endothelial cells and tubulogenesis, a response that does not happen with any of the GFs alone (33). HGF is also implicated in cutaneous wound pathologic neovascularization and exuberant granular tissue formation (4).

Platelet chemokines

Chemokines or chemotactic cytokines are proteins that induce cellular attraction. These peptides can be classified as housekeeping (constitutive) and inflammatory (inducible) chemokines, although some of them have a dual role (34). Inducible chemokines are crucial during inflammation. Produced at the site of tissue damage, they first act on white blood cells, producing migration and release responses (3). Constitutive chemokines are produced in tissues without the need of an inflammatory stimulus. These peptides are mainly responsible for several physiological processes including development, angiogenesis and apoptosis (34).

Chemokines act via seven membrane-domain receptors, which can be inducible or constitutive. PLTs contain two specific chemokines, PF-4 and β-TG. Other non specific chemokines released from PLTs include IL-8, growth-regulating oncogene-α (GRO-α), epithelial neutrophil-activating protein-78 (ENA-78), regulated on activation normal T expressed and secreted (RANTES), macrophage inflammatory protein 1-α (MIP1-α) and monocyte chemotactic protein-3 (MCP-3) (34). These chemokines play not only a role in PLT activation but also influence the nature of the leukocytic infiltration of injured or infected sites (3, 35).

PLTs are not the only source of PF-4, that is also produced by activated T lymphocytes and mast cells. The main receptor for PF-4 is chondoitin sulphate. This chemokine produces leukocyte chemotaxis, and also the firm adhesion of neutropils to endothelial cells. This strong cellular contact induces exocytosis of secondary granule contents (3). PF-4 is a powerful anti-angiogeneic peptide since it inhibits endothelial cell proliferation, migration and angiogenesis *in vitro* and *in vivo* by binding proteoglycans and interfering with the proteoglycan-bystander effect on growth factor activity. In addition, PF4 is able to interact directly with angiogenic growth factors such as FGF or VEGF and inhibits their interaction with cell surface receptors. This peptide may also activate cell surface receptors on endothelial cells and induce inhibitory signals for angiogenesis (3, 34). PF-4 inhibitions monocyte apoptosis and induced the differentiation of these cells to macrophages and histamine release from basophils (34).

Beta-thromboglobulins are three related peptides stored mainly in PLTs. These chemokines produce neutrophil chemotaxis and degranulation of primary and secondary granules. However in

late inflammation stage, β-TGs desensitize neutrophil degranulation and act as anti-inflammatory proteins. Both PLT specific chemokines produce inhibition of megakaryocytopoiesis (3, 34).

Platelet eicosanoids

Platelets release eicosanoids derived from the arachidonic acid that comes from membrane phospholipids. Thromboxane A_2 (TXA_2) is an important vasoconstrictor released during vascular injury. PAF is a chemotactic lipid that mediates leukocyte arrest and activation on endothelial cells or adherent platelets through a GPIIb-IIIa-dependent mechanism (15).

Platelet response

When injury occurs, a strong cellular interaction is triggered. This interaction produces different PLT responses including change of shape, internal transformation, granule secretion, formation of the primary haemostatic plug and clot retraction (14, 16). PLT surface molecules, such as integrins, regulate the capability for intercellular communication during the haemostatic plug formation, the inflammatory process and tissue repair (4). Kinetic expression is different for each type of PLT granule. First of all, α-granules release their contents because of a low intensity stimulus , later dense granules are activated and finally lysosomal granules release their proteolytic products (14, 18). This chain of events is known as PLT release reaction (2, 16, 18). It is important to point out that, *in vitro,* primary PLT aggregation is reversible and occurs without this release reaction. Secondary PLT aggregation is not reversible and induces the release reaction (18). Horse PLTs are especially susceptible to the ADP, collagen and PAF, which stimulate the release response (17).

Pathobiology of wound healing

Classically, the wound healing process has been arbitrarly divided into inflammatory, proliferative, angiogenic, epithelialization, contraction and matrix deposition and remodelation phases (1, 27). It is important to point out that all of these phases may occur simultaneously and that the general pathobiologic mechanisms can be adapted for all adult tissues including skin, muscles, tendons and joints.

Inflammatory phase

Inflammation is the most important defense reaction of the body against an insult or an aggression. It is designed to protect the body against excessive blood loss and invasion by foreign

substances. It also serves to prepare for the repair process that follows (36). Inflammation and repair are manifestations of the same process.They start almost simultaneously and coexist during all the inflammatory process. When injury occurs, there is a vasoconstriction of the arterioles which provides a provisional clot that prevents further blood loss. After a few seconds, vasodilatation occurs allowing the passage of cells, proteins and fluids into the wounded space (1, 27, 36). PLTs and fibrin provide a temporal matrix that will be replaced by granulation tissue in the next phase (1, 4). Vessels contract due to PLT induced mediators that modulate tone and permeability, resulting in stopping haemorrhage (14). They provide a provisional matrix of fibrin, fibronectin, and thrombospondin for the wound (1, 4). This matrix is a source of GFs and chemokines which are slowly released and that will act during all the wound healing process (4).

The coagulation cascade starts with the exposition of collagen, which activates blood factor XII (Hageman's factor), and the disruption of the endothelium, which activates factor VII and also stimulates the adhesion of platelets (via the thrombin pathway) (27). The coagulation cascade ends with the deposition of fibrin and the formation of a provisional clot that allows early cell migration. The coagulation cascade also activates fibroblast proliferation (via thrombin) and shields mitogenic and chemotactic factors from their natural inhibitors. The cascade of coagulation produces a myriad of inflammatory mediators such as bradikinin or C3a/C5a (anaphylotoxins) whose function is to activate the release of vasoactive mediators like histamine and leukotrienes from mast cells, macrophages, neutrophils and PLTs (36).

Activated PLTs release pro-inflammatory cytokines (interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)), and growth factors (PDGF, TGF-α, TGF-β, EGF, IGF, VEGF, and HGF), which stimulate leukocyte migration (27). TNF-α and IL-1 promote inflammation by activating the migration of phagocytic cells and their oxidative burst, increasing endothelial permeability, inducing adhesion molecules on the endothelium and intensifying their own production on cytokines and growth factors (1). Secretion of TNF-α and IL-1 is down-regulated by IL-6, which is produced by leucocytes, fibroblasts and endothelial cells (27).

The first cells to arrive at the inflammatory site are the neutrophils, probably because they are present in a higher number in the blood stream. They disappear three days later when macrophages become the most numerous cells. Crossing of leucocytes from blood to the wound surface begins with leukocyte margination (peripheral orientation of leucocytes). Margination allows the contact between the leucocytes and the endothelium and forms a stasis in blood called rolling. Rolling of neutrophils is a prerequisite for high affinity interactions (36). Low affinity neutrophil rolling is provided by P-selectin and L-selectin. The endothelium, inflammatory cells and PLTs express these molecules (1, 16).

Rolling serves to slow the intravascular neutrophil movement and to promote their exposure to soluble activating factors that stimulate adhesion and transendothelial migration. These factors are intracellular cell adhesion molecule type 1 (ICAM-1), vascular cell adhesion molecule type 1 (VCAM-1) in the endothelium and CD18, LFA-1, MAC-1, VLA-4 in the neutrophil (27). The expression of these molecules by the implicated cells is determined by chemokines such as fibrinopeptides, fibrinogen, fibrin lysin products, C5a, leukotriene-B4 (LTB_4) (from activated neutrophils), formyl methionyl peptides (from bacterial proteins), PAF, TNF-α, PF-4, PDGF, IL-8, growth-related proteins and melanoma growth stimulating activity (MGSA) (1, 27). Once adhered to the endothelium, leucocytes migrate from the blood stream to the wound surface by a process called diapedesis (36).

Monocytes are activated by monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1- alpha (MPI-1α), platelet derived endothelial cell growth factor (PD-ECGF), thrombin, TGF-β, collagen, elastin and fibronectin. Transmigration is mediated by interactions between ICAM-1 integrins, as well as platelet endothelial cell adhesion molecule type 1 (PECAM-1) on leukocytes and endothelium (27). Once outside, they migrate following a chemical gradient that depends on the population of cells involved, their state of activation, the leukocyte migration capacity, the nature of the vascular bed and the presence of chemotactic agents (the greater their concentrations, the faster the migration) (1). In the late stages of the inflammatory phase, there is a transition from inflammation to repair. At this point the most predominant cell is the macrophage instead of the neutrophil (36). Monocytes become macrophages by the stimuli of insoluble fibronectin, low oxygen tension, chemotactic agents (PF-4), bacterial lipopolysaccharides (LPS) and interferon (IFN) (27).

Macrophages lyse necrotic tissue but they also release growth and regulatory factors which are critical for the coordination of granulation tissue formation (PDGF, colony stimulating factor (CSF-1), TGF-β, TGF-α, FGF and IL-1). They form histiocytes or ephitelioid cells, attract fibroblasts into the wound and stimulate them to undergo maduration and collagen synthesis (1, 27).

Fibroplasia

Formation of granulation tissue begins at the end of the inflammatory phase. Fibroplasia is optimized in the acid medium provided by the accumulation of lactate from anaerobic metabolism and the macrophages are the cells most involved in the process of fibroplasia. They produce mediators that stimulate angiogenesis and fibroblast migration. Granulation tissue is necessary because it provides a surface through which epithelial cells can migrate and because it prevents infection (36). Macrophages, fibroblasts and endothelial cells form a type I and III collagen matrix, together with fibrin, fibronectin and proteoglycans rich in hyaluronic acid (HA), which allow cell

migration, act as a support mechanism for the deposition and orientation of collagen fibrils and bind connective tissue cells to the ECM (1, 27).

In the next stage, these fibrils will help fibroblasts to migrate by a process of contact guidance. Fibroblasts respond to chemokines and GFs released by macrophages and stimulate ECM synthesis. The proliferation of fibroblasts begins with the stimuli caused by the absence of neighbouring cells, EGF, TGF-α, TGF-β, FGF (expressed by the influence of TGF-β that is mitogenic for both endothelial and fibroblasts), heparin-binding epidermal growth factor (HB-EGF), PDGF, IL-6, IL-6F1, which are secreted by platelets, macrophages and keratynocites (27). TGF-β is secreted primarily by macrophages and platelets, and plays an important role because it regulates fibroblast proliferation, stimulates matrix production and inhibits its degradation by increasing protease inhibitor production (21, 22). In this way TGF-B promotes the formation of granulation tissue, attracts monocytes, macrophages, other inflammatory cells and fibroblasts and stimulates them to produce potent mitogenic peptides such as VEGF and FGF-1 (20). When fibroblasts are stimulated by GFs they secrete more TGF-β. Interestingly, this peptide has the function of inhibiting the cellular proliferation at this phase (21, 25).

After the temporary matrix is deposited, fibroblasts suffer a process of apoptosis or acquire smooth muscle characteristics and transform into myofibroblasts. Myofibroblasts have large actin bundles displayed longitudinally within the peripheral cell cytoplasm (27). TGF-β stimulates the differentiation of fibroblasts into myofibroblasts, creates other conditions necessary for contraction and enhances contractile forces (20-22). Fibroblasts migrate by following a gradient of chemotactic factors: complement metabolites, C5a, PDGF, PF-4, TGF-β, IL-4, and type I and III collagen fragments and fibronectin. This migration ceases when the edges become united and cells re-establish their original morphology and function and their desmosomal and hemidesmosomal attachements (27).

Angiogenesis

Angiogenesis develops together with fibroplasia. Angiogenesis starts with the disruption of the basement membrane by the action of collagenases and plasminogen activator secreted by the endothelial cells. Thanks to this, endothelial cells can migrate with cytoplasmic pseudopodia towards the site of the angiogenic stimulus (36). Endothelial cells produce fibronectin and collagen that form an ECM over which migration can be possible. Endothelial proliferation and differentiation are regulated by pericytes, cells that surround capillaries and migrate along them. Pericytes can reduce the angiogenic response by inhibiting the growth of adjacent endothelial cells. These new capillaries are more fragile and very permeable, so the new granulation tissue is edematous (1, 36). Molecules involved in angiogenic stimuli are FGF, TNF-α, IL-8, lactic acid,

biogenic amines, PD-ECGF, TGF-β, PAF, VEGF and PDGF (secreted by platelet, monocyte, and wound macrophages) (1, 27). FGF-1, TGF-β and VEGF favour the binding of endothelial cells to ECM and hence their migration (25, 29). PF-4 is a powerful anti-angiogenic chemokine (3). In normal conditions, there is a balance in the regulation of angiogenic and angiolitic peptides (25).

Epithelialization

Epithelialization is the slowest phase of the wound healing process. It begins 1 to 2 days after wounding and in this phase there is a reconstitution of the cells of the epidermis and other epithelia (36). There is a migration of keratinocytes from the edge of the cut epidermis across the defect, possibly thanks to the temporary dissolution of desmosomes and hemidesmosomes. Migration is produced thanks to the formation of pseudopodia (27). Molecules involved are EGF, KGF, TGF-β, which enhance the synthesis of epithelial cell-surface receptors for ECM components and IL-1 and TGF-α, which are also considered essential for epithelial migration and proliferation (1). Several GFs enhance proliferation, whereas TGF-β enhances the proliferative potential of epithelial cells (20, 21, 25). There is an inverse relationship between the epithelialized area and wound contraction: wounds showing more contraction have less epithelialization, and they are also affected negatively by persistent inflammation (36). The monolayer of cells becomes attached and differentiates into a stratified epidermis, but this process may take weeks to months (1, 27).

Contraction

By day 7 post wounding, the skin defect is reduced thanks to the centripetal movement of the surrounding undamaged skin. Myofibroblasts are the cells involved. Wound contraction determines the speed of secondary intention wound healing (36). The most important molecules affecting wound contraction are PDGF and IFN gamma (IFN-γ) (1, 20, 27). Myofibroblasts have connections with each other and with the ECM and they synthesize fibronectin and β_1 integrin receptors necessary for wound contraction (1). At the beginning new tissue is very thin. Later, cells start to proliferate and add thickness to the tissue. Wound contraction is greater in body regions with loose skin than in regions in which skin is under tension, such as the distal part of the limb in the horse (36). Inflammatory mediators such as prostaglandins, TNF-α, IL-6 and IL-1, inhibit contractibility (1, 27). Wound contraction is interrupted in three cases: when similar cells become in contact with one another (contact inhibition), when skin tension equals pulling forces of contraction or when exuberant granulation tissue prevents the wound's ability to contract. When myofibroblasts persist beyond the time required for wound closure, they may favour ECM accumulation and pathologic contraction, a condition leading to significant morbidity, particularly when it involves joints or body orifices (27, 36).

Matrix remodelling

The maturation phase is characterised by the decrease of the number of fibroblasts and reaching an equilibrium between collagen production and lysis (36). The balance between collagen synthesis and degradation during the remodelling phase depends on the simultaneous presence of matrix metalloproteinases (MMPs) and their natural specific tissue inhibitors (TIMPs) (1). MMPs denature and degrade collagen. Any disparity between MMP and TIMP may lead to abnormal resorption and delayed repair (27). Collagen becomes aligned parallel to the skin surface as a result of tensional forces produced by the wound edges. Tensile strength rises slowly up to only 75 to 80 % of the strength of normal surrounding tissue (36).

Are the chronic musculoskeletal pathologies non adequate processes of wound healing?

There are several studies that support the hypothesis that chronic musculoskeletal pathologies such as OA (37, 38) and tendinopathies (39, 40) are due to a non-adequate process of repair and inflammation resolution (37-40). In horses an up-regulation of catabolic cytokines (IL-1 and TNF-α) in tendons (41) and articular cartilage (37-38) and the aberrant apoptosis of their resident cells, tenocytes (42) and chondrocytes (43, 44) have been found. Theories to explain the ethiology of these degenerative diseases include ageing and repetitive trauma (37-40). However, they do not specify which event occurs first, catabolic cytokine up-regulation or premature apoptosis of resident cells. Any of the two pathophysiological mechanisms may unchain impaired repair, which worsens with ageing and excessive physical activity (37-40). In the horse, chronic musculoskeletal pathologies should be considered as chronic inflammatory processes with impaired biological control. IL-1 and TNF-α self-perpetuate ECM degeneration by up-regulating MMPs and prostaglandins and down-regulating TIMPs. This catabolic picture is deleterious for resident cells and does not allow the arrival or differentiation of mesenchymal stromal cells (MSCs) for replacement; thus poor quality ECM is produced and tissues are more prone to be re-injured (38, 40, 43).

Methods for preparing autologous human platelet concentrates

Whitman et al (45) introduced the use of autologous PCs in maxillofacial surgery for sinus floor augmentation. This PC was called autologous platelet gel and was considered as a substitutive source for autologous fibrin glue. Fibrin glue was extensively used as a scaffold for bone graft anchorage and to improve haemostasis at the surgical site (46). Fibrin glue is a two-component mixture in which concentrated fibrinogen, factor XIII (fibrin-stabilizing factor) and fibronectin are added to thrombin, calcium chloride and an inhibitor of fibrinolysis to form a fibrin

clot (45). The fibrinogen can be derived from a random donor, a single-donor cryoprecipitate or autologous plasma (46). The use of this substance has been discouraged since commercial homologous cryoprecipitates may produce allergic reactions (45) and viral infections (47). In addition, the cost of producting fibrin glue is very high and autologous fibrin glue preparations are time consuming (3 days) and require adequate laboratory facilities (45).

Figure 2.4. PRGF kit®. This method is very simple, but requires a strict aseptic management.

Whitman et al (45) suggested the preparation of an autologous platelet gel from 450 ml of whole blood mixed with citrate phosphate dextrose as anticoagulant using an apheresis autotransfusion system, which can be used in the surgical room. Using a similar technique, Marx et al (6) reported the effects of a PC (platelet rich plasma (PRP)) in 88 elective cancellous cellular marrow bone graft reconstructions of mandibular continuity defects of 5 cm diameter or greater randomly assigned in two groups, control and treated with PRP. The results of that study showed that PRP treated grafts statistically (p=0.005) evidenced a radiographic maturation rate two times faster than control grafts (6). It is important to point out that the mean number of PLTs concentrated in the studies by Whitman et al (45) and Marx et al (6) was $750x10^3$ PLTs/µl approximately. The activation of both PCs was induced with bovine thrombin and 10% calcium chloride (6, 45).

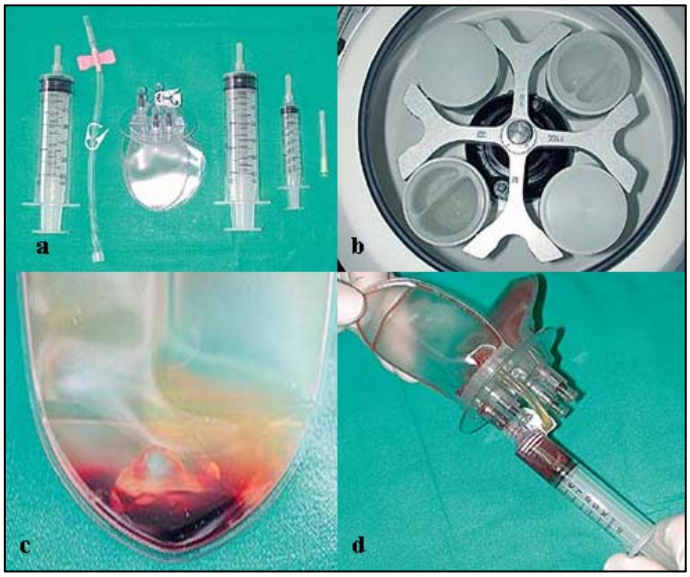

Fig. 2.5. Platelet Concentrate Collection System (PCCS®, Biomet-Merck, Bridgend, UK). **a)** Kit for blood collection. **b)** Centrifuge. c) Platelet concentrate obtained by centrifugation. **d)** Platelet concentrate is packed in a plastic syringe.

Anitua (7) reported a simple method for preparing PCs, known as platelet rich in growth factors or (PRGF) (PRGF kit®, CAC Medicale San Antonio, Vitoria, Spain) (**Fig 2.4**). The method for preparing PRGF is tremendously simple and cheap in comparison with the apheresis method. A simple centrifuge machine, 5 ml citrated tubes, 1 butterfly catheter are all what is needed to prepare PRGF (48). Anitua (7) demonstrated positive effects on patients, which required dental extraction for therapeutic purposes. Twenty patients were randomly asigned to one of two groups of ten: one group was treated with PRGF and the other acted as control. The PRP treated patients had 100% of re-epithelization and 80% of bone regeneration in comparison with the non PRP treated patients, in which re-epithelization was delayed and the osseous defects were only refilled with connective tissue (7). Anitua's PRGF technique concentrated a mean lower number of PLTs (400-500x10^3 PLTs/µl) (48) than the apheresis technique (6, 45). During PRGF preparation calcium chloride is the only substance used for platelet activation (7, 48). It is necessary to point out that bovine thrombin is expensive and is not free of side effects such as allergic reactions (4).

After Anitua's tube method was described, many semi-automated devices for concentrating human platelets have been developed. Some of these specially customized kits or systems include: Platelet Concentrate Collection System (PCCS®, Biomet-Merck, Bridgend, UK) (**Fig. 2.5**), Platelet Concentrate Collection System II (PCCS®II, Biomet-Merck, Bridgend, UK), Gravitational Platelet System (GPS™, Biomet-Merck, Bridgend, UK) (**Fig. 2.6**), Curasan Kit® (PRP Kit, Fa. Curasan,

Kleinostheim, Germany), Smart PreP System™ (Harvest Technologies, Mu-nich, Germany) (**Fig. 2.7**), SmartPReP®2 *APC*+™ (Harvest Technologies, Munich, Germany) (**Fig. 2.7**), Friadent Schütze PRP Kit® (Friadent Schütze. Viena, Austria), Secquire PRP System® (PPAI Medical, FL, USA) (**Fig. 2.8**), amongst others. These PLT concentrating systems provide higher PLT levels (>1000x10^3 PLTs/μl) (49-56) than some apheresis procedures (6, 45) and the PRGF method (48)(see **Table 2.2**). All the techniques used to prepare PCs (apheresis automated systems, semi-automated buffy coat devices and tube method) present advantages and drawbacks (6,7, 45, 49-57). There is not yet an ideal method or device for concentrating platelets.

Table 2.1. Some methods used for preparing platelet concentrates in human medicine. Comparison of some cellular and molecular aspects

Method	Platelets x10^3/μl	Leukocytes x10^3/μl	TGF-β_1 (ng/ml)	PDGF-AB (ng/ml)	IGF-I (ng/ml)	Reference
Apheresis	270-1408	0.1-17.4	221	117.5-125	84.2	50, 57
Tube	513.6	6.5	73.3	47	78	48
PCCS®	1073-2209	14.15	289.5	156.7	78	50,
GPS®	1601	ND	120	ND	ND	56
Curasan Kit®	1075-2519	14.8	499.8	295.2	ND	50, 52
Smart PReP™	1227	19.2	77.2	208.3	72.8	53
Friadent-Scütze PRP Kit®	1440.5	21.6	196.8	251.6	91.4	53

PC: Platelet concentrate; TGF-β: Transforming growth factor beta; PDGF: Platelet derived growth factor; IGF: Insulin-like growth factor. ND: Non disponible data

The apheresis system requires high technology and experienced personnel. This technique is not applicable for small clinical settings and requires a large volume of blood (>450 ml) in comparison with the other two techniques described (6, 45, 57). The most important advantage of this technique is its low risk of bacterial contamination during PCs preparation (58). PCs obtained by the apheresis technique have been extensively assessed for their platelet collection efficiency and quantity of some growth factors (see **Table 2.2**) (57).

The buffy coat systems have important technical advantages in comparison with the apheresis and the manual methods. A high number of PLTs and GFs are collected-concentrated when these devices are used (*see* **Table 2.2**) (49-56). The risk of bacterial contamination is lower

(58) than with the manual method (48) and they can be used in small clinical settings. However, these devices also concentrate a high number of leukocytes and they are expensive (49-56). It is important to point out that the exact role of leukocytes in PCs intended for regenerative objectives has not been established, but it is thought that high numbers of inflammatory cells could be detrimental for some treated tissues (54).

Fig. 2.6.Gravitational Platelet System (GPS^{TM}, Biomet-Merck, Bridgend, UK). a) Blood is deposited in a special recipient using the red cap. b) Blood is centrifuged in a specially designed centrifuge. d) The blood has been separated and the platelet concentrate is ready for its use.

The manual (tube) method is a simple and inexpensive technique for PC preparation. However, it requires strict aseptic management to avoid bacterial contamination (**Fig 2.5**) (48). This technique concentrates a lower number of PLTs and GFs (*see* **Table 2.2**) than the apheresis (52) and the buffy coat methods (49-56). With this technique, lower numbers of leucocytes are collected when compared whith other techniques (48). It is important to point out that the clinical use of autologous PCs obtained with the aforementioned methods, has obtained good clinical results, independently of the number of PLTs and leukocytes concentrated using with each technique (4-7). However, it is thought that the higher the PLT concentration obtained the better the tissular response (59). It is possible that this assumption derives from experimental observations in animal models, where a greater osseous proliferative and osteointegrative response was observed when >1000-1500 PLTs/μl were added to peri-implant osseous defects (60).

Figure 2.7. a-b) PreP System™ and its centrifuge **c-d)** SmartPReP®2 *APC*+™ and its centrifuge (Harvest Technologies, Munich, Germany).

Clinical use of platelet concentrates in human medicine

PCs are widely used in dentistry and oral-maxillofacial surgery (4-7). In fact, the popularity of the use of PCs in other fields of human surgery is due in part to the positive results obtained with the use of this substance in dentistry (4). PCs have been used to treat skin ulcers in diabetic patients (61) and as an adjuvant in plastic and reconstructive (8, 9), ophthalmologic (62) and orthopedic surgery (4, 10, 11). Autologous PCs are especially useful for soft tissue and bony reconstruction in plastic surgery. It has been noticed that the use of PCs decreases operation time, eliminares the need for drains and pressure dressings and decreases the incidence of complications, infections and of hospitalization time (8). Plastic surgeons have observed that PCs have anti-inflammatory, bactericidal, regenerative and haemostatic properties (4).

PCs are being used for several orthopedic surgery procedures including: lumbar spinal fusion (11), promoting healing of complicated bone fractures (combined with MSCs) (63), during total joint replacement (4), arthroscopic reattachment of traumatic osteochondral defects (10), Achilles tendon repair and intra-articularly to treat severe cases of osteoarthritis or refractory rheumatoid monoarthritis (R Soler unpublished data). PCs are also used for retinal repair in patients

with severe grade 4 idiopathic full-thickness macular holes, since they increase cellular proliferation during the healing of retinal wounds (62).

Figure 2.8. Secquire PRP System®.

The use of PCs has gained great popularity in human medicine (4). Unfortunatly, there is a lack of controlled clinical trials that would allow to exert a critical evaluation about the real advantages of the use of autologous PCs. The vast majority of human clinical publications about the use of PCs agree that these substances have beneficial effects on the wound healing process (59). However, there is also controversial data (especially from studies performed in *in vitro* systems and animal models) about the benefits of the use of PCs. The particular animal models used may play a role. A higher concentration of PLTs may aid human wound healing, whereas other animal species may heal well even without additional PLTs, making it more difficult to show a benefit of PCs in these models. The techniques used to concentrate PLTs in the various studies may influence the success. Some isolation techniques may contribute to early and premature platelet degranulation. However, one cannot rule out the possibility that the effect of PC on bone healing may be minimal (64).

Effects of PCs in experimental conditions

Many studies have been conducted to determine the anti-inflammatory and regenerative properties of PCs in induced craniofacial defects of laboratory animals (revised in 64). The effects of platelet supernatants have also been evaluated *in vitro* using different types of tissue explants and cells such as cartilage and chondrocytes (65), bone and osteoblasts (66), tendon and tenocytes (33), skin, periodontal ligament (4) and mesenchymal stromal cells (MSC) (67).

Effects of platelet concentrates on bone tissue

PCs improve bone healing and regeneration by promoting the differentiation of MSCs to osteoblasts and increasing the metabolic capacity of osteoclasts. In addition, they decrease the number or the effect of osteoclasts (66). GFs contained in PLTs, such as PDFG, TGF-β and IGFs play a paramount role in bone healing and homeostasis. PDGF induces proliferation of osteoblasts and bone collagen degradation (20). TGF-β may regulate the response of cells to PGDF and initiate bone formation (21). IGF-I up-regulates osteoblast activity and proliferation. This factor is of pivotal importance in osseous ECM synthesis (4).

Effects of platelet concentrates on cartilage

PCs can be useful for the treatment of different cartilage articular pathologies, including traumatic osteochondral fragmentation, osteoarthritis and rheumatoid arthritis. There is a considerable amount of information about the potential therapeutic effects of some GFs (20) that are present at supraphysiological doses in PCs (48-57). For example, IGF-I and TGF-β can increase the metabolic activity of chondrocytes and thus increase cartilage ECM synthesis (68, 69). IGF-I is also mitogenic for chondrocytes (68), whereas TGF-β stimulates the differentiation of synovial membrane MSCs to chondrocytes (69). These peptides possess a strong anti-inflammatory activity, since they can down-regulate catabolic cytokines. In addition, PDGF and bFGF induce chondrocyte anabolism and PDGF increases the vascularization of meniscus (70).

Effects of platelet concentrates on tendons and ligaments

PCs seem to be useful for the treatment of soft tissue musculoeskeletal pathology because they promote the vascularization of the tissues affected. TGF-β, PDGF, VEGF and HGF, all present in PCs, produce intense neovascularization of tendons and ligaments. This new blood supply

improves the metabolic activity of tenocytes (33). In addition, IGF-I and bFGF produce proliferation of tenocytes and increase ECM synthesis (20).

Effects of platelet concentrates on skin wounds

All the GFs contained in PCs play a central role in wound healing (1, 27) previously described in this review.

Methods for preparing autologous equine platelet concentrates

To date, two techniques for obtaining equine PCs have been described: apheresis and buffy coat methods (12, 71). An additional technique in order to improve the efficiency of collection of equine platelets after using a conventional method of filtration has also been reported (71). The aforementioned techniques are good, but they present technical and economical restrictions for many equine practitioners. For example, the apheresis method can only be performed in a specialized laboratory (6, 45, 12). On the other hand, the buffy coat method is very expensive (71). Both techniques for concentrating equine PLTs have been evaluated for PLT, leukocyte and levels of some GFs (**Table 2.3**) (12, 71).

Clinical use of platelet concentrates in equine medicine

An homologous commercial equine platelet gel obtained by apheresis and mixed with vitamin C (Lacerum®, BeluMedX, AR, USA) has been proposed for limb wound treatment in horses. The effect of this product was evaluated in one horse with experimentally induced limb skin defects. The PRP-treated defects healed faster than the non PRP-treated skin lesions.The results of this study were encouraging; however, its design of the study was not well controlled (12). There are anecdotic reports about the possible therapeutic applications of PCs in equine medicine, including their peri-lesional injection in tendon and ligament injuries and also for articular degenerative pathology. However, to date there are no equine studies published about the potential therapeutic or deleterious effects of PCs in horses.

Potential problems and risks of the clinical use of platelet concentrates

Different questions arise on the possible problems or risks associated with the use of autologous PCs (4, 73). For example, in human beings with thrombotic problems, PCs should not be used close to large blood vessels (4, 73, 74), since PCs have prothrombotic factors such as chemokines (74), tisular factor or IL-1 (75). This condition is not likely to be a problem in horses treated with autologous PCs, since thrombotic disease, different from verminous embolism, is not frequent in this species.

Table 2.2. Techniques used for preparing equine autologous platelet concentrates. Comparison of some cellular and molecular aspects.

		Technique		
Variable	**Whole blood**	**Buffy Coat method***	**Apheresis method**	**Apheresis method after filtration**
PLTs $x10^3/\mu l$	165	1472	490-855	2172
WBCs x $10^3/\mu l$	6.3	32.5	33.7	61.2
$TGF\beta_1$ ng/ml	5.5	15.3	7.4-23.6	57.9
$TGF\beta_2$ ng/ml	1.2	1.0	4.3	ND
PDGF-AB ng/ml	ND	ND	7.4	ND
IGF-I ng/ml	171.5	107.4	183.4	ND

*Secquire PRP System (PPAI Medical, FL, USA). WBCs: White blood cells. For keys see **table 2.2**. Data adapted from references 12 and 71.

Conclusion

The use of autologous PCs has an incredible popularity in human oral-maxillofacial and plastic surgery, since the clinical results obtained are positive and encouraging (4, 6, 45). However, some skeptical clinicians do not recommend the use of PCs based in some results observed in animal studies, where homologous PCs were evaluated (64), instead of autologous PCs, for osseous regeneration or skin wound healing (59). These two points of view will remain unsolved until blind

randomized controlled clinical trials are performed on a large sample of patients. Furthermore, it is mandatory to choose an adequate animal model for evaluating the potential beneficial or detrimental effects of these substances on experimentally induced lesions in bone, tendon and ligament, and in natural diseases common to animals and humans, such as degenerative musculoskeletal diseases (76).

The horse represents an ideal model to evaluate the potential beneficial or detrimental effects of autologous PCs on different experimentally induced lesions (e.g: collagenase induced tendonitis and desmitis, LPS induced synovitis or ostechondral fragmentation induced OA (77). The same applies in naturally occurring equine chronic musculoskeletal pathologies with similar pathophysiological mechanisms than the diseases suffered by human beings, such as OA, tendinopathies or desmopathies (37-40). In addition, a large volune of whole blood can be used to prepare equine autologous PCs from the same horse (of different cellular and molecular characteristics) without detrimental effects. This does not occur in small laboratory animals (e.g: minipigs or rabbits) and even in small or medium sized dogs. However, the use of horses as an animal model for human disease implies serious economic and ethical restrictions that make the use of this species difficult in musculoskeletal applied research (77). Finally, it is obvious that clinical well controlled (blinded and randomized) studies in horses with chronic musculoskeletal pathology using autologous PCs can be useful to determine the predictable effects of these substances in human beings with the same pathologies.

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III. CLINICAL EVALUATION OF THE EFFECTS OF AUTOLOGOUS PLATELET CONCENTRATES IN HORSES WITH SEVERE JOINT DISEASE

CLINICAL EVALUATION OF THE EFFECTS OF PLATELET CONCENTRATES IN HORSES WITH SEVERE JOINT DISEASE

Summary

The clinical effect of the intraarticular injection of a platelet concentrate (PC) in 7 horses with severe joint disease (4 with osteoarthritis and 3 with osteochondrosis) was evaluated. The degree of lameness (DL), joint effusion (JE), and clinical follow-up were recorded. Three injections of the PC were performed at two week intervals. Horses were evaluated before each injection and two months after the last treatment. Clinical follow-up was conducted during 1 year. Count of platelets, leucocytes, and determination of TGF-β_1 levels per ml of the PC were performed, as well as leukocyte count, cytology and protein levels in synovial fluid (SF). The PC produced a statistically significant improvement in both the DL and JE ($p<0.05$). The most marked improvement was observed 2 months following the last treatment and apparently persisted up until 8 months later. A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg of TGF-β_1 per ml of the PC were obtained. No adverse clinical signs resulted from this treatment. Despite the seemingly positive effects of this substance, the clinical use of PCs cannot be recommended until further studies with higher number of cases and longer follow up can be undertaken.

Introduction

Joint disease can be produced by several causes, such as repeated traumatic injury, joint instability, infection and developmental problems of the endochondral ossification (osteochondrosis (OCD)), amongst others. All of these factors can potentially produce severe damage of the articular cartilage. If the inciting cause is not diagnosed and treated timely, an irreversible degenerative process of the cartilage is produced and the final result is known as osteoarthritis (OA) (6).

Biochemichally, OA may result from the final imbalance between anabolic and catabolic peptides which stimulate the production and the remodelling of extracellular matrix (ECM) components of the articular cartilage (6, 20). Joint homeostasis depends in many ways on the adequate expression of various GFs. Some of these have anabolic effects (6, 20). Transforming growth factor beta 1 (TGF-β_1) , (11, 14, 31), insuline like growth factor type 1 (IGF-I) (13, 19, 23), IGF-II (10), and platelet derived growth factor (PDGF) (30) all have been shown to promote both chondrocyte proliferation and ECM synthesis (20). Other peptides such as, interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) produce chemotaxis, leucocitic degranulation, expression of

proinflammatory mediators, such as prostaglandin E_2 (PGE_2), leucotriene B4, NO and matrix metalloproteinases (MMPs) which degrade articular cartilage (20, 21, 24, 25).

Numerous studies document the *in vitro* effects of GFs in equine chondrocytes and cartilage (10, 11, 13, 15, 23). The results of these studies support the possibility of using purified extracts (20) or of manipulating the genes that encode the production of these peptides (12) in order to manage equine OA. Platelet concentrates (PCs) are an autologous source of many GFs, such as TGF-β_1, PDGF, and IGF-I (2, 8, 24, 28, 29) and other molecules that modulate inflammation and tissue repair (2, 8). This substance has been used in maxillo-alveolar reconstruction (5), and plastic surgery (4). There is a case report documenting its use in the successful treatment of a non traumatic cartilage avulsion in a soccer player (22). Furthermore, the use of a PC in a gel form has been reported in an experimental study involving skin healing mechanisms in a horse (8). Excellent results were reported but the investigation was limited to one horse only.

There has been an increase in the popularity of PCs in human (2, 4, 5) and equine (8, 24) medicine but little is known about the biological behaviour of this substance (24, 28, 29). In the present clinical study the therapeutic effects of an autologous PC administered within the joint in a group of horses affected with severe joint disease was investigated. The effects of this PC were evaluated clinically by documentation of its effects on degree of lameness (DL), joint effusion (JE), and some synovial fluid (SF) parameters.

Material and methods

This pilot, clinical, prospective study was approved by the Board of the Veterinary Medical Teaching Hospital of the Universitat Autònoma de Barcelona, Spain. All the owners signed a sheet of consent authorizing treatment and were informed of the possible risks of joint injection or complications derived from PC injection. All the observations and determinations were performed by two separate clinicians.

Patients- Seven horses with signs of severe joint disease were included in the study. They were of different breeds and sex and their average age was 6 years (range 1.2-15 years). A total of 10 joints were evaluated, since 3 of these horses had bilateral joint compromise (**Table 3.1**). Case selection criteria included detailed musculoskeletal examination and diagnostic procedures including radiography, ultrasound examination, regional anesthesia and arthroscopy in 2 horses.

Table 3.1. Signalment and clinical signs of the horses in this study.

Horse No	Breed	Sex	Age (years)	Discipline	Joints affected	Diagnosis	Previous treatment	PC (ml/art)
1	Arabian	Male	7	Endurracing	Fetlocks	OA	IA steroids	10
2	Haflinger	Male	15	Dressage	Left Coffin joint	OA	IA steroids and HA	10
3	French mountain horse	Female	8	Carriage	Left tibiotarsal and proximal intertarsal joints	OA	NSAIDs	15
4	Warmblood	Male	6	Jumping	Right medial femorotibial joint with a medial meniscal lesion	OA	NSAIDs	20
5	Hannoverian	Filly	1.4	Intended use, jumping	Stifles	OCD	Arthroscopic Surgery IA steroids and HA	20
6	Spanish Sport horse	Filly	1.2	Intended use, jumping	Stifles	OCD	Arthroscopic Surgery	20
7	Holsteiner	Male	3.5	Jumping	Left tibiotarsal joint	OCD	No previous therapy	15

OCD: Osteochondrosis. OA: Osteoarthritis. HA: Hialuronic acid. IA: intraarticular

Clinical study design

Lameness examination- The lameness exam was scored from 0 to 5 according to the parameters of the AAEP (1). Only trot in a straight line on a hard surface was considered. A one minute long flexion test was performed in 4 adult horses and in the fillies (*n:* 2) only in the last exams (since they had not been handled) but they were not included in the statistical analysis. Lameness after flexion was graded from 0 to 3, where 0= negative response - 3= markedly positive response.

Ultrasonographic examination- An ultrasonographic evaluation of the affected joints was performed. Joint surface, degree of synovial effusion, thickness and appearance of synovial membrane were evaluated. Degree of JE was graded from 0 to 3, where 0= joint normal in appearance - 3= severe effusion and synovial membrane thickening. Ultrasonographic measures were always performed in the same anatomical point in each patient by the same clinician.

Synovial fluid analysis- Synovial fluid analysis and cytology was performed in those events when fluid was obtained prior to joint injection.

Clinical follow-up. Clinical follow-up of each patient was conducted during 1 year.

Preparation and analysis of the PC- Whole blood was aseptically drawn of the jugular vein of each patient via 23G butterfly catheter (Terumo, Belgium), and deposited in 3.2% (wth/vol) sodium citrate tubes with capacity for 5 ml (BD Vacutainer^{TM} (9NC 0.129M) systems, Uk). Afterwards, they were centrifuged at 120 g during 5 minutes. The first supernatant plasma fraction (50%), adjacent to the buffy coat, was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged at 240 g during 5 minutes and the 25% from the first fraction was obtained. This last fraction was placed into sterile syringes and activated with calcium chloride (B. Braun Medial SA, Europe, (4.5 mEq/5 ml)), using 50 µl per ml of PC. A fraction of the PC (2 ml) of each patient was analyzed for platelet and leukocyte count and TGF-□$_1$ levels determination. Cell count was performed by a flow cytometry hematology system (ADVIA 120, Bayer, USA), and TGF-β_1 levels were determined by a human commercial ELISA kit (R&D System, USA).

Schedule of the PC treatment- The affected joint(s) of each horse were prepared aseptically for injection. The horses were sedated with an intravenous bolus of detomidine (Domosedan, Pfizer) and butorphanol tartrate (Torbugesic, Fort Dodge Laboratories Inc). Horses were injected 3 times at 2 weeks intervals. A complete clinical examination was performed before each PC administration and 2 months after the last joint injection. The amount of PC used in each patient was subjectively determined depending on the type and size of the joint, and weight of each patient (Table 3.1). The horses were generally kept at a lower level of exercise during treatment and for 2 weeks following the last injection.

Statistical analysis- Values of DL and JE were expressed as means with their respective ranges. Data was analyzed with a Kruskall Wallis test. It was assumed that in the case statistical significance was found a Wilcoxon paired test of would be performed. The level of significance for both tests was $p \leq 0.05$. Synovial fluid parameters and PC values were presented in a descriptive manner.

Results

The 7 horses included in the study suffered severe joint disease. Four horses had OA (1 mare had OA of the left tibiotarsal and proximal intertarsal joints, 1 horse had OA of the left coffin joint, 1 horse had bilateral OA of the anterior fetlocks), 1 horse had OA of the medial femorotibial joint with a medial meniscal lesion, 2 fillies had bilateral OCD of the stifle, an 1 horse had OCD of the left tibiotarsal joint. The horses with OA had been treated with rest for 2 months up to 1 year, corticosteroids and hyaluronic acid. The 2 fillies with stifle OCD had undergone bilateral arthroscopic procedures with debridement of 40% of the lateral trochlear ridges of the femur.

Table 3.2. Lameness degree and joint distension scores of the horses in the study.

Parameter	Value			
	Initial	**Before 2nd injection**	**Before 3rd injection**	**Final evaluation**
Degree of lameness	1.1 a Range 0.5-2	0.8 a,b Range 0-1.5	0.6 b,c Range 0-1.5	0.35 c,d Range 0-1
Degree of joint effusion	2.25 a Range 2-3	1.75 b Range 1-2.5	1.5 c Range 1-2.5	0.875 d Range 0-1

a-c : Values with different letters in a row are significantly different statistically ($P<0.05$).

The fillies were included in the study because there were fragments remaining in the joint and a second surgery was not within the economic reach of the owners. One of these animals had been operated 3 months before the treatment with the PC and had been turned out in the paddock a month prior to treatment. The other one had had surgery 6 months prior to treatment with the PC. The 2 fillies and the colt with OCD were kept turned out. One mare was kept in training for carriage

but at a lower intensity. Two horses were ridden lightly and finally 1 horse in training for endurance was maintained at the same level of work despite our recommendations of reducing work load during the treatment period.

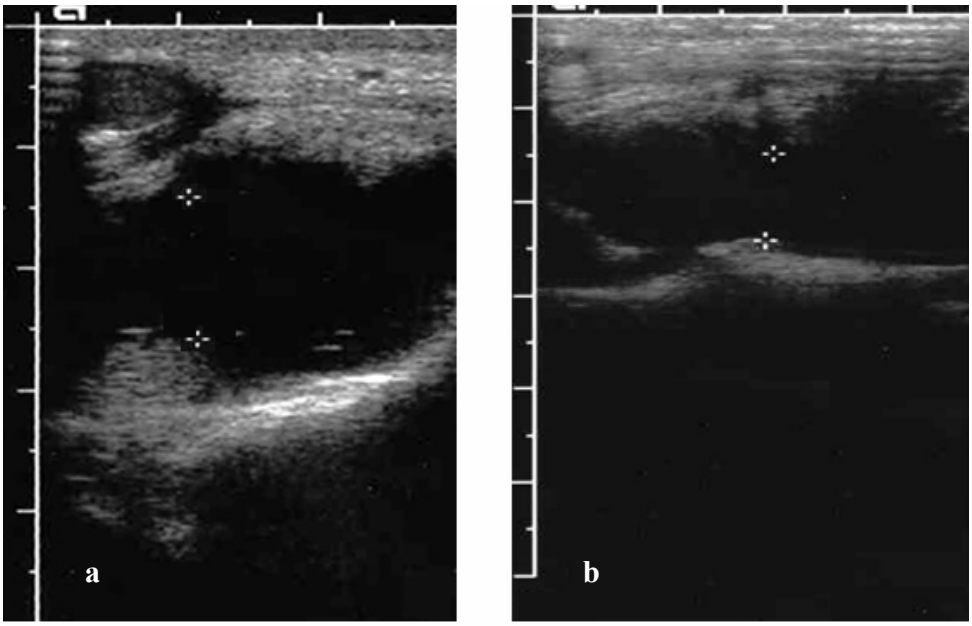

Figure 3.1. Ultrasonographic longitudinal sections of the dorsomedial recess of the tibiotarsal joint (2cm below the medial malleolus) of the horse number 7 with osteochondrosis. Note the decreasing of the synovial effusion. **a)** Image before the treatment with platelet rich plasma. **b)** Image after the third injection.

The lameness scores improved after intraarticular administration of PC and this was statistically significant (p= 0.048) in the horses in the study. The improvement of the lameness score was gradual and was most significant after the third treatment and 2 months after (**Table 3.2**). Flexion tests could not be performed at the beginning on the 2 younger not very handled fillies, but the trend was of generalized improvement in all of the flexion tests performed. There was a statistically significant improvement in the JE (p= 0.00043) during the treatment and 2 months after the last injection (**Fig 3.2**). Joint fluid cytology performed when fluid could be obtained samples revealed a predominance of mononuclear cells (98%). It was not possible to perform statistical evaluation of SF samples (*see details in* **Table 3.3**). A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg of TGF-β_1 per ml of PC were obtained.

No adverse clinical signs resulted from this treatment. However, a moderate, transient synovial effusion was observed after the two first PC injections in a filly (horse No 4) with OCD of

the stifle and in patient No 6 with OA of the stifle. The horses with OA maintained their final lameness score for about 8 months after the last PC injection and, then showed a gradual increase of the DL. The two fillies and the colt with OCD remain without lameness, but they have not yet been trained at high intensity.

Table 3.3 Description of synovial fluid parameters from the joints of some of the horses treated in this clinical study.

Parameter	Value		
	Initial (n:3)	**Before 2nd injection (n: 4)**	**Befote 3rd injection (n:3)**
Total Protein (g/dL)	1.73± 0.5	1.4 ± 037	1.73 ± 0.4
Leukocytes (x10^3cel/µlL)	0.57± 0.06	0.78 ± 0.2	0.53± 0.06

Values are expressed as means ± standard deviation. n: number of joints

Discussion

This is the first report, to our knowledge, of the use of an autologous PC for the intraarticular treatment of joint disease in the horse. We describe an easy, reliable and inexpensive technique for obtaining an equine PC. A schedule for treatment with a PC is proposed. However, this fact is merely empiric and it is based in a schedule of treatment used for the management of severe inflammatory arthropathies in human beings (R. Soler, unpublished data). We decided to begin this pilot clinical study because there is scientific and anecdotic evidence about the positive effects of this substance on human cartilage and chondrocytes (14, 22).

The improvement in both DL and JE in the horses of this study could be explained by the effect some GFs have on inflammation and tissue repair (27). During joint disease, especially OA, IL-1 and TNF-α promote the expression of nuclear factors like, nuclear factor kappa-beta (NF-κB) (17) which upregulate the genes that encode secondary proinflammatory peptides such as, IL-6, IL-8, IL-12, chemokine, eicosanoids, NO and MMPs (21, 25, 26). From a clinical point of view the sum of all these substances can be manifested as pain and synovial effusion in horses affected with chronic joint disease (6). One could think that the local administration of PC may have had an analgesic and antiinflammatory effect in the patients of this study by possibly inhibiting the

expression of these nuclear factors or by blocking the effects of their metabolic catabolites (2, 27). Nevertheless, this hypothesis remains to be validated experimentally.

Joint effusion is produced by an increase in blood flow and capillary permeability with protein leakage which produce interstitial edema and an overall increase in joint fluid. Moderate synovitis may have beneficial effects on joint nutrition, but severe effusion affects joint function by creating articular surface incongruency, instability and joint pain (3). The increase in joint distension after each injection with PC could have been produced by the fact that a relatively large volume of PC was being administered, by the osmotic effect of the proteins injected in the joint space (3, 6), by the biochemical effects of this substance in the joint environment (22) or perhaps by the chemotactic effects of the leukocytes present in the prepared platelet concentrate (24, 28, 29). However, this phenomenon was transient since the joint fluid effusion reverted to a significant decrease in joint distension before each subsequent treatment and at the end of the study (**Table 3.2**).

In this study a lower concentration of platelets (250 ± 71.8 x 10^6 platelets/ml) was obtained in comparison with the equine values reported for apheresis method by Carter et al (8) (490 x 10^6 platelets/ml) or by Sutter et al (24) (855 x 10^6 platelets/ml) or for buffy coat method (1472 x 10^6 platelets/ml) (24). However, we obtained higher TGF-β_1 levels (12515 ± 2443 pg/ml) than those reported by the apheresis method by Carter et al (8) (7480 ± 1315 pg/ml), but lower TGF-β_1 levels in comparison with Sutter et al (24) (23600 pg/ml). Using the buffy coat method, Sutter et al (24), reported a total of 1472 x 10^6 platelets/ml and 15300 pg/ml of PC. Again, if we compare the results obtained with our technique, it is possible to note that the platelet number is higher in that study (24) in relation with our results. However, TGF-β_1 levels obtained with both techniques were very similar. It is important to note that many not well defined factors can influence the final GFs levels of platelet concentrates obtained by different techniques (8, 24).

Ideally one would try to minimize the number of leukocytes injected into joints. With our technique an inferior number of leukocytes (8.68 ± 3.78 cells x 10^6/ml) was obtained in comparison with the results described by Sutter et al (24) for apheresis (33.7 leukocytes x 10^6/ml) and buffy coat (32.5 leukocytes x 10^6/ml) methods. However, the importance of leukocyte type and number in platelets concentrates remains not well understood and actually researchers do not know if the presence of these inflammatory cells is responsible or not of the some beneficial and detrimental effects of PC in the tissues. Another important factor to consider in the use of PC is the method used for activation. In our study calcium chloride was used to stimulate platelet activation. In another study (8) platelets were activated with thrombin, which although not specified, was most likely to be bovine in origin. The use of this protein can be associated with allergic reactions (2). In this

sense the method described here is safer and less costly than in other methods where purified bovine thrombin is utilized.

Concentrations of GFs in PCs are high when compared to the levels required to stimulate equine cartilage anabolism *in vitro*. Concentrations of 5000 pg/ml of TGF-β_1 produce chondrocyte mitosis and stimulate ECM synthesis (11, 14, 29). This same action has been observed with doses of 200-500 ng/ml of IGF-I (13, 23) and of 25-50 ng/ml of IGFII (10). One must remember that GFs i*n vitro* could act very differently to what is observed *in vivo* (20). Perhaps supraphysiological concentrations of GFs, (e.g.: TGF-β_1) can be found in these PCs, could control catabolic and inflammatory processes occurring in joint diseases, like OA. Recently, in another study (*see* **Section V**) it was found that the top plasma fraction (50%) obtained during the first centrifugation to concentrate platelets presents a similar concentration of TGF-β_1 than the fraction (PC) used in this study. The number of leukocytes present in this platelet fraction was lower in comparison with the number of leukocytes present in the platelet fraction used in this study. We hypothesize that this fraction may be better for treatment of joints, since the leukocytes could be responsible of the transient joint effusion observed in some of the patients of this study. However, this hypothesis should be further validated.

The short and limited effect of the vast majority of therapeutic agents used to combat joint disease and particularly OA is a very important problem (9, 18). Horses suffering OA need periodic treatment. Very few agents modify the course or progression of the disease and some may actually accelerate joint destruction (7, 9, 18). The sum of the analgesic plus the seemingly prolonged antiinflammatory effect observed in these horses treated with autologous PC in the joint make us think this could be a remission inductive therapy (16). However, our study had serious limitations that cannot lead us to conclude that PC may really being used for the treatment of equine joint disease. It should be considered that number of the cases that we evaluated was small and not representative. Furthermore, we did not have age and disease matched controls. It is known that rest and time usually diminish clinical signs of joint disease (6, 7, 18) but it may be worthy to note that our study the patients we included had had clinical signs of joint disease for at least 3 months and up to a year prior to treatment and except in 2 cases the horses went back to the same level of activity that they had prior to treatment. Nevertheless, from our positive clinical observations we think that intraarticular PC injection may be a promising new instrument in the treatment of joint disease in the horse. However, this therapy can not be recommended until *in vitro* studies had been performed and more clinical cases have been assessed. Ideally, a prospective randomised double blind clinical study would be necessary to validate these initial clinical observations.

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IV. CLINICAL EVALUATION OF THE EFFECTS OF AUTOLOGOUS PLATELET CONCENTRATES IN HORSES WITH SOFT TISSUE MUSCULOSKELETAL INJURIES

CLINICAL EVALUATION OF THE EFFECTS OF PLATELET CONCENTRATES IN HORSES WITH SOFT TISSUE MUSCULOSKELETAL INJURIES

Summary

The efficacy of a PC in 7 horses with soft tissue musculoskeletal injuries namely: superficial digital flexor tendon (SDFT) tendinopathy and desmopathy of the suspensory ligament (DSL) was evaluated. Initial degree of lameness (DL), flexion test response (FT), ultrasonographic images, and long term clinical follow-up were documented. Three injections of a PC were performed at two week intervals. Horses were evaluated before each injection, two months after the last treatment and 1 year after. Platelet counts, WBC counts, and determination of TGF-β_1 levels per ml of PC were performed. Two patients had acute SDFT tendinopathy, 1 horse had chronic SDFT tendinopathy, 1 horse had chronic bilateral forelimb proximal DSL (PDSL), 2 horses had chronic bilateral hindlimb PDSL, 1 horse had bilateral forelimb desmitis of the branches of the suspensory ligament (BDSL). All the horses in this report presented clinical and statistical ($p < 0.05$) decrease of DL and response to FT. Ultrasound appearance improved in the horses with SDFT lesions, but remained the same in the horses with DSL. The horses with acute SDFT tendinopathy returned successfully to competition level without reinjury. The horse with chronic SDFT tendinopathy relapsed. Horses with forelimb and hindlimb PDSL returned successfully to competition level without reinjury. A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg of TGF-β_1 per 1 ml of the PC were obtained.

Introduction

Injuries of tendons and ligaments heal slowly and inefficiently. Once a lesion is produced, these structures do not recover their original biomechanical properties. It has been hypothesized that factors like fatigue, hypoxemia, hyperthermia, and overstrain can unchain a degenerative process of the extracellular matrix (ECM) of these tissues (17), including changes in the collagen molecules ratio with a shift to type III collagen (*Col-III*) production in detriment of *Col-I* synthesis (11), and death of resident cells (20). However, the exact pathophysiologic mechanism that produces these changes remains poorly understood (13, 27).

The presence of catabolic cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 alpha (IL-1α) and IL-1β in biopsies of equine tendons suffering from chronic tendinopathy has been reported (18). It has been shown that the exogenous administration of IL-1β

to human tenocytes upregulates the expression of IL-1β and IL-6, cyclo-oxigenase 2 (COX-2), and matrix metalloproteinases (MMPs). It is known that all these substances have catabolic and inflammatory properties which have detrimental effects on ECM and resident cells of many tissues, including tendons and ligaments (32). The negative effects of IL-1 or TNF-α on connective tissue could be down-regulated by some growth factors (GFs) (33). Studies *in vitro* have demonstrated the beneficial properties of transforming growth factor beta (TGF-β) (2, 23, 24,), insulin-like growth factor type 1 (IGF-I) (9), vascular endothelial growth factor (VEGF) (35), fibroblastic growth factor (FGF)(23, 34), epidermal growth factor (EGF), and platelet derived growth factor (PDGF) (23, 34) on tendon and ligament metabolism of different animal species.

Table 4.1 Clinical history and sex, breed, age and aptitude of the patients inthe study

Horse No	Breed	**Sex**	**Age (years)**	**Discipline**	**Affected Limb**	**Diagnosis**	**PRP (ml/lesion)**
1	Crossbreed	Female	10	Jumper	LF	Acute SDFT tendinopathy	5
2	Crossbreed	Castrated male	9	Pleasure	RF	Chronic SDFT tendinopathy	7
3	Crossbreed	Male	11	Jumper	RF	Acute SDFT tendinopathy	7
4	Warmblood	Male	11	Dressage	L&RF	Chronic PDSL	10
5	Angloarabian	Castrated male	6	Jumper	L&RH	Chronic PDSL	10
6	Trotter	Male	5	Trotter	L&RH	Chronic PDSL. BDLS of the medial branch of the RH	10-4
7	Arabian	Male	14	Pleasure	L&RF	Chronic bilateral BDLS. Bilateral Fetlock OA	4

RF: Right forelimb. LF: Left forelimb. RH: Right hindlimb. LH: Left hindlimb. SDFT: superficial digital flexor tendon. PDSL: Proximal desmitis of the suspensory ligament, BDSL: branch desmitis of the suspensory ligament. OA: osteoarthritis.

Moreover, Dahlgren et al (10) described a positive action of IGF-I in a collagenase induced tendonitis model of the equine superficial digital flexor tendon (SDFT). The results from these

studies suggest the possible benefit of using GFs as a complementary treatment of soft tissue musculoskeletal injuries, either using pure recombinant proteins or gene therapy (6, 9, 12).

Platelet concentrates (PCs) are a natural and autologous source of some GFs, especially TGF-β_1, TGF-β_2, PDGF, VEGF, EGF, FGF and of other molecules that modulate inflammation and the tissue repair process (2, 3, 30). PCs have been used successfully in human medicine, for several purposes, including alveolar-maxilar reconstruction (2, 7), plastic (5), and orthopedic surgery (2). There are anecdotic reports about the use of PCs for the treatment of Achilles tendonitis in two athletes and for cartilaginous avulsion in a football player (26). Recently, Anitua et al (3) documented the positive effects of a PC on human tenocytes. In that study, PC produced tenocyte proliferation and upregulation of two angiogenic peptides, VEGF and hepatocyte growth factor (HGF).

In the present study, the results from the treatment with an autologous PC in 7 horses with tendon and ligament injuries are reported. The objectives of this report were to describe the possible beneficial efficacy and clinical safety of an autologous PC injection in horses with soft tissue musculoskeletal lesions.

Material and methods

This pilot, clinical, prospective study was approved by the board of the Veterinary Medical Teaching Hospital of the Universitat Autònoma de Barcelona, Spain. The owners were informed about the nature of the experiment and the possible complications derived from the PC injection. All the observations and determinations were performed by two separate clinicians.

patients- Seven horses of different sex, age, breed and aptitude were included in this study. The average age of the horses was 9.14 years (range 3-14 years). A total of 11 limbs were treated as some of the horses had a bilateral lesion (**Table 4.1**). All the patients were evaluated seven days before being treated. A complete lameness examination was done including perineural and intraarticular regional anesthesia when necessary in order to localize the lesion in the limb. A complete ultrasonographic and lameness examination was performed before every treatment, two months after the last injection and 1 year after.

Design of the study

Lameness examination- The lameness exam was scored from 0 to 5 following the AAEP criteria (1). Where 0= Horse presents a normal gait – 5= Horse is not able to bear weight on the affected limb. A one minute flexion test was done and the results were classified with a qualitative scale from 0 to 3, where 0= negative result 1=slight increase in lameness 2= moderate increase in lameness 3= severe increase in lameness

Table 4.2. Ultrasound evaluation and classification of the horses´ superficial digital flexor tendon lesions, before, during and at the end of the treatments.

	Ultrasound lesion evaluation									
Horse No	**Before 1st treatment**	**C**	**Before 2nd treatment**	**C**	**Before 3rd treatment**	**C**	**Two months after last treatment**	**C**	**1 year after last treatment**	**C**
1	Circular and lateral lesion at 2A region of 2 cm in length. Represents 10% of the CSA of the tendon.	IV	The lesion is smaller and less extended (1cm). Fibers are more organizad.	III	There is no objective ultrasound difference between the lesion and the rest of the tendon.	II	Similar to the last evaluation.	II	Similar to the last evaluation	II
2	Several small, hypoechoic lesions and a small anechoic lesion lateral aspect of 2A region, extending about 4 cm in length.	V	There is a moderate change in the increase in echogenicity of the lesions.	III	There is just a small hypoechoic lesion in 2A region.	III	There is no evidence of ultrasonographic lesion in the tendon. Slight thickening was evident	I	There is no evidence of ultrasonographic lesion in the tendon.	I
3	Hypoechoic lesion placed medially in region 2A . It represents 20% of the CSA. Perimeter of the lesion is 17,3 mm^2.	IV	There is an increase of the echogenicity of the lesion and a decrease of its size. Perimeter of the lesion is 14,8 mm^2.	III	There is an increase of the echogenicity of the lesion and good alignment of the fibers at the site of the lesion.	III	Close to normal echogenicity pattern and normal fiber orientation at the lesion site.	II	There is no evidence of ultrasonographic lesion in the tendon.	I

C: Lesion category classification. CSA: Cross sectional area

Ultrasound evaluation- A modified Rantanen et al's ultrasound classification (24) for equine tendon and ligament injuries was used. Clinical signs (heat, inflammation, lameness, sensitivity, thickness of the anatomic structures) and clinical history of each patient was evaluated together with the results of the ultrasound exam. Ultrasonographic parameters used in this study included: cross sectional area (CSA), maximum injury zone (MIZ), MIZ percentage respect to the healthy portion of ligament or tendon, echogenic pattern of the lesion and orientation of the fibers in both longitudinal and transverse sections. Clinical and ultrasonographic examination of each horse were combined in order to create a qualitative scale of lesions in six categories from 0-V, where 0= tendon ultrasound image normal and no lameness or any clinical sign associated with tendonitis or desmitis is present – V= Presence of very severe anechoic lesions, large increase in the CSA-MZI, severe disorganization of the fibers in longitudinal and cross section, evident clinical signs with a moderate or severe degree of lameness (24).

Clinical follow-up- Clinical follow-up of each patient was conducted during 1 year. The treatment with the PC was considered successful when the horse returned to the same pre-injury training or competition levels without relapse of the injury in the follow-up time.

Preparation and analysis of the PC- Whole blood was aseptically drawn of the jugular vein of each patient via a 23G butterfly catheter (Terumo, Belgium), and deposited in 3.2% sodium citrate tubes with capacity for 5 ml. It was then centrifuged at 120 g during 5 minutes. The first supernatant plasma fraction (50%), adjacent to the buffy coat, was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged at 240 g during 5 minutes and the 25% from the first fraction was obtained. This last fraction was placed into sterile syringes and activated with calcium chloride (4.5 mEq/5 ml), using 50 µl per ml of PC. An average of 5 ml of PC was obtained from every 75 ml of whole blood. A fraction of PC (2 ml) of each patient was analyzed for platelet and leukocyte count and TGF-β_1 concentration determination. Cell count was performed by flow cytometry (ADVIA 120, Bayer, USA), and TGF-β_1 levels were determined by a human commercial ELISA kit (R&D System, USA).

Schedule of the PC treatment- The affected area of the limb of each horse was prepared aseptically for injection. The horses were sedated with an intravenous bolus of detomidine (Domosedan, Pfizer) and butorphanol tartrate (Torbugesic, Fort Dodge Laboratories Inc). The PC was injected 3 consecutive times at two week intervals. The amount of PC used in each patient was subjectively determined depending on the type and size of lesion, and weight of each patient (**Table 4.1**). The horses were generally kept at a lower level of exercise during treatment and for 2 weeks following the last injection.

Table 4.3. Ultrasound evaluation and classification of the horses´ suspensory ligament injuries, before; during and after the treatments.

Horse No	Ultrasound lesion evaluation									
	Before the 1st treatment	**C**	**Before 2nd treatment**	**C**	**Before 3rd treatment**	**C**	**Two months after last treatment**	**C**	**1 year after last treatment**	**C**
4	LF: Thickening of the SL origin in 1A region (10.8 mm in longitudinal section) and periostitis of 2nd and 4th MTT in the 2B region.	II	LF: Decrease of the longitudinal section of the origin of the SL (10.5 mm)	II	LF: no changes	II	LF: no changes.	II	LF: no changes.	II
	RF: No changes in the SL are observed (8.3 mm in longitudinal section).	I	RF: no changes.	I	RF: no changes	I	RF: no changes	I	RF: no changes	I
5	Thickening of both SL origin in both hindlimbs.	II	No changes in any limb.	II	No changes in any limb.	II	No changes in any limb.	II	No changes in any limb.	II
6	Thickening of both SL origin in both hindlimbs and hypoechogenic lesion in the medial SL branch of the RHL	IV	No changes in SL origins. Increasing echogenicity of the medial branch the SL	III	No changes in the SL origins. No lesions observed in the SL branch	II	No changes from the last evaluation	II	No changes from the last evaluation	II
7	Heterogenicity and severe hypoechogenicity of both branches of the SL of both FL and periligamentous inflammation.	V	There is an increase of the homogenecity of the SL branches. The Sl lateral branch of RF presents a very small central lesion still.	III	No lesions observed.	I	Ultrasound normal	I	Ultrasound normal	I

For key see Tables 1 and 2**.**

Statistical Analysis- The analgesic effect of the PC was statistically analyzed. Only the patients with soft tissue lesions were evaluated (1-6). Data from degree of lameness and flexion test obtained before each injection with PC and two months later were analyzed with a Kruskall-Wallis test. It was assumed that in the case of finding significant differences in time, a non parametric pairwise comparison Wilcoxon test would be done. A $p \leq 0.05$ value was established as significant for both tests. Data from the ultrasound exam and the values obtained from the PC were presented just in a descriptive way.

Results

Of the evaluated horses, 2 patients had acute SDFT tendinopathy, 1 horse had chronic SDFT tendinopathy, 1 horse had chronic forelimb proximal desmitis (PD) of the suspensory ligament (PDSL), 2 horses had chronic hindlimb PDSL, 1 of these patients also had desmitis of the medial branch of the suspensory ligament of the right hindlimb, and 1 horse had bilateral forelimb desmitis of the branches of the suspensory ligament (BDSL) accompanied by bilateral fetlock osteoarthritis (**Table 4.1**). The animals with SDFT lesions presented initial ultrasound scores between IV-V (median V) (*see details in* **Table 4.2**). The horses with desmitis of the SL had initial ultrasound scores between I-IV (median II) (*see details in* **Table 4.3**). At the beginning of the study the horses presented a slight grade of lameness and a moderate response to flexion test (**Table 4.4**). PC produced a statistically significant decrease of degree of lameness (p=0.0053) and of the response to flexion (p=0.0062) during the study. The improvement in both parameters was most evident at the end of the study. The cosmetic appearance of the injured area was excellent already after the first injection in the cases of SDFT.

Serial ultrasonography examinations showed improvement in the score of the lesions, especially in the horses with SDFT tendonitis (**Table 4.2**). The horses with either forelimb or hindlimb bilateral PDSL did not change their ultrasonography category. However, they improved the scores of both degree of lameness and response to flexion test. Horse number 7, with a severe bilateral BDSL showed both a marked ultrasonographical and clinical improvement (**Table 4.3** and **Fig. 4.1**). The horses with PDSL finished with a median ultrasound lesion score (median II), but with a decrease in the range of the classification (I-II) (**Table 4.2**).

The horses with acute DSFT tendinopathy returned to complete exercise and to competition level 4.5 months after last PRP injection without reinjury at 1 year of the last PC treatment. Patient number 3 with chronic DSFT tendinopathy presented a relapse of the lesion 3 months after the last PC treatment when galloping was started. This horse returned to work again 6 months after relapse and did not return to competition level. The patient is now used as a master school horse.

Table 4.4. Scores of the degree of lameness and flexion test of the patients of the study.*

Variable	Value			
	At the beginning	**Before 2nd PC injection**	**Before 3rd PC injection**	**Last evaluation (2 months later)**
Degree of lameness	0.9a Range 0-2	0.64a Range 0-1.5	0.44a,b Range 0-1.5	0. b Range 0-1
Flexion test	1.85 a Range 0-3	1.14 b Range 0-2	0.71 b Range 0-2	0. b Range 0

* The horse number 7 was not included. [a-c]: Values with different letters in a same row are statistically significant ($P<0.05$). PC: Platelet concentrate

Horse number 4 with chronic forelimb PDSL returned to pre-injury level 2 months after the last PC injection without reinjury at 1 year of follow-up. Horse number 5 with one year duration bilateral hindlimb DPLS and horse number 6 with the same pathology returned to competition level 6 months after the last PC injection without re-injury at 1 year of follow-up. Patient number 7 with bilateral BDLS improved from its soft tissue lesions, but it is still lame because of the fetlock joint osteoarthritis. A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg of TGF-β_1 per 1 ml of PC were obtained.

Discussion

This is the first report, to our knowledge, about the use of an autologous PC for the treatment of musculoskeletal soft tissue injuries in horses, although we are aware that some equine clinicians have used PC before for soft tissue injuries, the results have not been published. In the study presented here, we describe an easy and inexpensive technique for obtaining PC. This pilot clinical study was performed on clinical equine patients because there is scientific and anecdotic evidence about the positive effects of this substance on human soft tissues (2, 7, 5), including tendon and ligament injuries (3, 26).

Tendon and ligament pathologies are frequent in human and equine sports medicine. The pathologic process that affects these structures could be considered as degenerative in nature (11, 22). Traumatic events should be considered as a separate entity. However, many tendon or ligament acute (traumatic) lesions could be related to reinjury of a chronically active lesion or to failure of scar tissue within the structure (13, 17, 27). Maybe, the SDFT lesions presented in the horses number 1 and 2 could be included into this pathologic condition. It is known that horses with the same classification of SDFT tendon lesion (grades IV-V) to the horses of this study are very prone to reinjury, despite having followed a controlled exercise programme and conventional treatment. Normally, 9-12 months of rest are recommended as a part of the treatment of these patients (14, 21). We found that the 2 horses with acute SDFT tendinopathy treated with PRP returned to competition level in half of the expected time and did not relapse in comparison with literature documented controls treated with conventional treatments, such as polysulfated glycosaminoglicans, hyaluronan or fumarate of beta aminopropiontrile (BAPN) (14, 21).

Horses with SDFT tendinopathy are prone to develop reinjury of the initially affected tendon or to overstress and injury of the contralateral SDFT (14, 21). Horse number 3 reinjured the SDFT above the original area of lesion despite of PC treatment. It is likely that this treatment is less useful in chronic lesions where scar tissue already exists and there has been permanent loss of tissue elasticity. Another explanation at a cellular level for this situation could be the exaggerated apoptosis that has been seen in this pathology. Recently, Hosaka et al (20) demonstrated that apoptosis plays an important role in the pathophysiology of this disease. It is possible to think that a if there is a reduction in the population of tenocytes and if these cells aditionally are metabolically impaired by the action of catabolic cytokines (18), mainly TNF-α (19), they will not react in a positive fashion to the addition of any purified GF or PC. In this sense, the use of mesenchymal stromal cells accompanied with a PC (28) or other delivery systems of GFs could be an option for the treatment of this pathology in advanced chronic phases (27). In addition, it may be reasonable to perform microsplitting of the affected area with a needle to stimulate bleeding and a vascular accesss to the center of the lesion together with a PC injection. This was not performed in these horses but this procedure could be evaluated in a prospective clinical study. Peripheral blood contains mesenchymal stromal cells that have the potential capacity to transform into tenocytes if adequately stimulated (28). Platelet concentrates contain many peptides that could potentially stimulate the differentiation of these cells (3, 8, 30), in addition to an environment with linear tension, like in tendons or ligaments. Although, to our knowledge, molecular changes associated with catabolic cytokines and apoptosis have not been described in the SL, it could be possible to assume that the same occurs in ligamentous tissues.

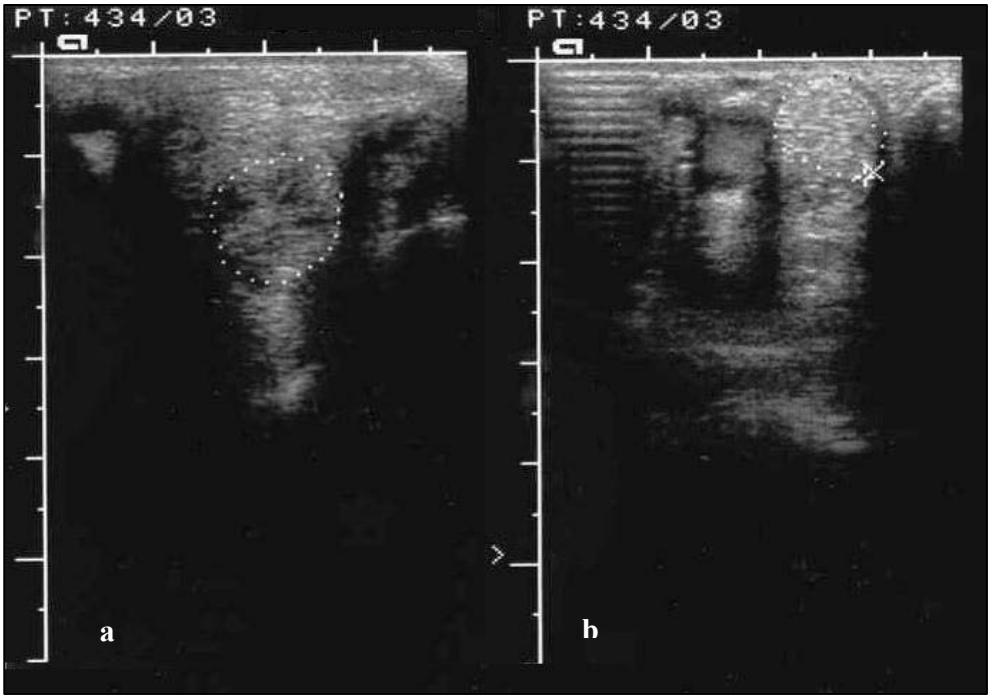

Figure 4.1. Ultrasonography image of the lateral branch of the suspensory ligament of the right forelimb of an 14 year old Arabian gelding horse (patient Number 7), with chronic bilateral forelimb desmitis of the branches of the suspensory ligament. Note the diffuse hypoechogenecity, and the loss of the fiber pattern orientation before PRP treatment **(a).** The same structure three weeks after of the first PRP injection **(b).**

The prognosis of SL injuries depends on the extent, the affected area and limb. PDLS has a better prognosis in forelimbs than in hindlimbs. Horses with forelimb PDSL can attain pre-injury training level after a three month programme of rest followed by controlled exercise (15, 16). Horse number 4 of the series presented here with forelimb PDLS, returned to pre-injury exercise level two months after the last PC injection. In this horse the effect of time and rest could have influenced recovery. However, when considering clinical history and the positive follow-up of horses 5 and 6 with chronic severe bilateral hindlimb PDSL, we think that the treatment with PCs could be useful for the management of this frustrating pathology. It is important to note that PDSL of the hind limbs can be totally limiting for an athletic career and that some of the radical treatments that have been tried, fasciotomy with or without neurectomy of the lateral branch of the plantar nerve will fail since most of the horses with this pathology will continue to have a degenerative process of the SL that could even lead to its rupture (15, 16).

Desmitis of the branches of the SL is more often described in the forelimbs than in the hindlimbs and is normally associated to a chronic process (15). The ultrasonographic lesions

observed from horse number 6 were very severe. This patient's ultrasonographic scores improved dramatically after even the first PC treatment. It is known that this type of lesion heals very slowly (more than 18 months) and patients can take more than 9 months to return to normal performance (15, 16). Unfortunately, this horse had also osteoarthritis in both forelimb fetlock joints, and despite its excellent ultrasonographic progress the horse remained lame (**Fig 4.1**).

Some of the most relevant clinical observations from the perilesional treatment of these soft tissue injuries were the following: even after the first injection of PC the cosmetic appearance of the injured area was excellent, meaning there was less heat, pain, swelling and lameness; there seemed to be a better response in injuries that were presented within a short period after injury. The improvement of the ultrasonographic scores was most evident and remarkable for acute suspensory ligament branch injuries. The chronic proximal hindlimb suspensory ligament desmitis cases showed no improvement in their ultrasonographic appearance although it has to be pointed out that these horses did not show anechoic lesions, just fiber disorganization and thickening of the origin of the ligament these horses though did improve clinically and actually went back into work with no relapse of the injury. The results observed from the patients of this study, especially in horses number 5 and 6, could indicate that PRP has a powerful and long-term analgesic and antiinflammatory effect. PCs have not only an important amount of TGF-β_1, but it has important levels of other angiogenic (FGF, VEGF), proliferative (PDGF, IGF, FGF and EGF) and anabolic (IGF-I) GFs of paramount importance in the wound repair process (2, 3). Peptides like TGF-β_1 or IGF-l have potent anti-inflammatory effects, since they downregulate the expression of nuclear proinflammatory factors, such as NF$\kappa\beta$ (33). This factor is activated for IL-1 or TNF-α during equine tendinopathy (18, 19), and is responsible of the upregulation of several secondary catabolic substances, like MMPs, prostaglandin E_2, and NO, amongst others (32, 33).

The main limitation of this clinical report is the small number of cases documented and the different pathologies. Nevertheless, from our clinical observations we think that PC injection could be a highly promising new instrument in the treatment of desmopathies and tendinopathies in the sport horse. To date, more than 50 racing horses with chronic soft tissue musculoskeletal pathology have been treated with the autologous PC. Preliminary results of this research are encouraging since patients treated with PC-C have a better ultrasonography score and return to the competition level faster than control group. In addition, no complications derived from the use of PC-C have happened during more than 120 PC-C injections (Abellanet et al., unpublished data). However, this therapy can not be recommended until more clinical cases have been assessed and ideally a larger prospective randomized clinical study with a longer follow up would be necessary to validate these initial clinical observations.

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V. EVALUATION OF SINGLE AND DOUBLE CENTRIFUGATION TUBE METHODS FOR CONCENTRATING EQUINE PLATELETS

EVALUATION OF SINGLE AND DOUBLE CENTRIFUGATION TUBE METHOD FOR CONCENTRATING EQUINE PLATELETS

Summary

The aim of this study was to evaluate single and double centrifugation tube methods for concentrating equine platelets. Whole blood samples were collected from clinically normal horses and processed by use of single and double centrifugation tube methods to obtain 4 platelet concentrates (PCs): PC-A, PC-B, PC-C and PC-D, which were analyzed using a flow cytometry haematology system for hemogram and additional platelet parameters (mean platelet volume, platelet distribution width, mean platelet component concentration, mean platelet component distribution width. Concentrations of transforming growth factor beta 1 (TGF-β_1) were determined in all the samples. Platelet concentrations for PC-A, PC-B, PC-C and PC-D were 45%, 44%, 71% and 21% higher, respectively, compared to the same values for citrated whole blood samples. TGF-$\beta_1$concentrations for PC-A, PC-B, PC-C and PC-D were 38%, 44%, 44% and 37% higher, respectively, compared to citrated whole blood sample values. In conclusion the single and double centrifugation tube methods are reliable methods for concentrating equine platelets and for obtaining potentially therapeutic TGF-β_1 levels.

Introduction

The healing of wounds (either in soft or hard tissues) is regulated by several cell types and by a cascade of peptides known as cytokines or growth factors (GFs). When an injury occurs, GF secretion by platelets and macrophages is induced, and the inflammation-healing process is initiated (1). Although numerous GFs are involved, transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) play key roles in wound healing (2, 3). TGF-β_1 favours the chemoattraction of monocytes and macrophages, and a combined attraction and proliferation of fibroblasts (1, 3). These peptides also regulate the transcription of extracellular matrix proteins, including fibronectin, collagen and glycosaminoglycans (1, 4).

Platelet α-granules contain a variety of GFs, some of which are specific to platelets (PLTs), whereas others are also produced by other cells, such as leukocytes (2). The main GFs secreted by platelets include: TGFβ_1, TGFβ_2, PDGF-AA, PDGF-BB, PDGF-AB, insulin-like growth factor type I (IGF-I), epidermal growth factor, and hepatocyte growth factor (5, 6). Platelet concentrates (PCs) are an important source of autologous GFs (5, 6). Attention has been given in human medicine to the use of PCs for alveolar-maxillary reconstruction (7), plastic (8) and orthopaedic surgery (9). It has been suggested that the supraphysiological GF concentrations present in this substance could act in a positive fashion by accelerating wound

healing, decreasing the inflammatory reaction and promoting the regeneration, rather than repair, of the affected tissues (2, 6). However, the clinical use of PCs in the treatment of arthropathies (*see* **Section III** of this thesis) and soft tissue musculoskeletal lesions in horses (*see* **Section IV** of this thesis) have been reported, as well as an experimental report of its use in equine skin lesions (10). The preliminary results observed in these studies seem to be encouraging.

Platelet concentrates can be obtained by means of at least three general methods: the tube, buffy coat and apheresis methods (revised in **chapter III** of this thesis). Several commercial devices for preparing PCs using the buffy coat procedure have been developed and evaluated for use in human medicine (11-22). However, only one commercial device for obtaining PCs has been experimentally assessed in horses (23). The apheresis method for preparing equine PCs has also been described (10, 23). The buffy coat and apheresis methods for obtaining PCs in horses have been analyzed for platelet and leukocyte counts as well as for $TGF\beta_1$, $TGF\beta_2$, PDGF-AB, and IGF-I levels (10, 23). However, the tube method has been used for preparing PCs, especially in human dental surgery (12, 15, 22). The advantages of this technique are the low cost and the minimal technical requirements in comparison to the other two general methods described above (13, 14, 16-21). However extreme care should be taken to avoid bacterial contamination when this technique is performed (22). Furthermore, this technique has not been evaluated in horses.

Until now, studies published about human (11-22) and equine (10, 23) PCs proposed for wound healing have focused on determining both the cellular population, mainly platelets, WBCs, and RBCs, and the levels of TGF-β_1, PDGF and IGF-I. In this sense, some general positive correlations between the number of platelets and TGF-β_1 levels, and between the total WBCs count and PDGF-AB levels in these platelet preparations have been found (11, 23). However, there is lack of information about the distribution and proportion of each type of leukocyte collected and about other parameters related to these cells and platelets.

A commercially available flow cytometry hematological system (ADVIA 120 Analyzer, Bayer Lab, NY, USA) can be useful for evaluating equine PCs, since it performs the WBC differential (24) and measures different platelet parameters, such as mean platelet volume (MPV), platelet distribution width (PDW), mean platelet component concentration (MPC), mean platelet component distribution width (MPCDW), number of large platelets, and platelet clump counts, among others (25-27). The MPV represents the size of the platelet. The PDW is an indicator of the average size of the platelet population. The MPC is useful in determining the density of platelets by their refractive index. This parameter is associated with platelet degranulation; values decrease when platelets release their granular content. The MPCDW indicates the variation in the density of platelets; this parameter increases when circulating platelets are present in both activated and non-activated form (25, 26).

In the present study, single and double centrifugation tube methods to obtain four different equine PCs (PC-A, PC-B, PC-C and PC-D) were evaluated. PC-A and PC-B are derived from single tube centrifugation technique and PC-C and PC-D are derived from double centrifugation tube method. Our hypothesis was that these PCs would differ in the number of platelets and leukocytes, level of platelet activation and TGF-β_1.

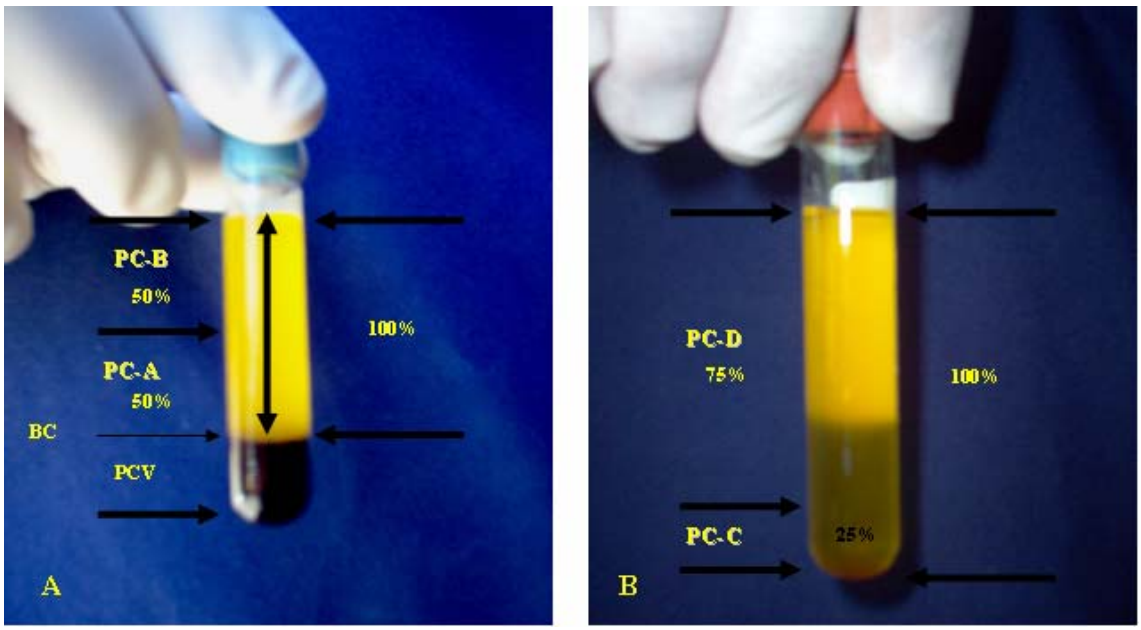

Figure 5.1. Picture **A** represents the first two platelet concentrates obtained by the single centrifugation tube method. Picture **B** represents the second PCs obtained by the double centrifugation tube method. BC= Buffy coat. PC= Platelet concentrate. *See* **Tables 5.1-5.2** for keys.

Material and methods

Horses- Twenty six clinically normal horses were included in this study, 11 mares, 1 stallion and 14 geldings aged from 3 to 12 years (mean age 6.9 ± 2.3 years), and of different breeds. All the animals lived at a riding school near the Large Animal Veterinary Medical Teaching Hospital of the Universitat Autònoma de Barcelona and were fed and managed under the same conditions.

Blood collection- One hundred and twenty five ml of whole blood was collected from each horse from the jugular vein using a 23 gauge butterfly catheter (Terumo Europe NV, Belgium) and deposited in 25 tubes of 3.8 % sodium citrate (BD Vacutainer systems, Plymoth, UK). Blood from one randomly chosen tube was used to perform a hemogram and subsequently for plasma preparation. The other 24 tubes were also randomly chosen for the preparation of platelet concentrates either for the single (8 tubes -40 ml-) or double (16 tubes -80 ml-) centrifugation tube method. The average time between blood extraction and the initiation of sample processing was 30 minutes.

Single centrifugation tube method- Forty ml of citrated blood (8 tubes of 5 ml each) were centrifuged (Rotofix 32, Germany) at 120 x *g* for 5 minutes. Two different fractions of PCs were obtained: PC-A and PC-B. We arbitrarily classified PC-A as the 50% fraction closest to the buffy coat, and PC-B was classified as the 50% fraction of the supernatant above PC-A (*see* **Fig. 5.1a**). Each fraction was obtained with a 9 cm, 18 G spinal needle (BD Yale Spinal, Madrid, Spain) attached to a 20 ml plastic syringe. Five ml of each PC were deposited separately in 10 ml polypropylene tubes (Deltalab, S.A., Rubí, Spain).

Double centrifugation tube method- Eighty ml of citrated blood (16 tubes of 5 ml each) were centrifuged at 120 x *g* for 5 minutes. After the PC-A fraction of each tube was obtained, 20 ml of this fraction were deposited in two 10 ml polypropilene tubes. This fraction was centrifuged once again at 240 x *g* for 5 minutes. The centrifuged PC-A sample was arbitrarily divided into two fractions. PC-C was designated as the lower 25% fraction of the centrifuged PC-A sample and PC-D was designated as the remaining upper 75% fraction. (*See* **Fig 5.1b**). Five ml of PC-C and PC-D were deposited separately into 10 ml polypropilene tubes and used in the study.

The relative forces (*g*) and time of centrifugation used in this study were determined after preliminary studies (data not shown) done in our laboratory facilities.

Platelet and leukocyte counts- A complete hemogram was performed for each sample in duplicate (whole blood, PC-A, PC-B, PC-C, and PC-D) using a flow cytometry hematology system (ADVIA 120 Analyzer, Bayer Lab, NY, USA) Hematological parameters evaluated included: packed cell volume (PCV), platelet count, leukocyte (WBC) count, as well as the relative and absolute numbers of neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cell (LUC) counts, values for MPV, PDW, MPCDW, MPC, large platelet count, and platelet clumps (aggregates).

Activation of Platelet concentrates- Once the hemogram had been performed, 250 µl of a 10 % calcium chloride solution (Braun Medical, Barcelona, Spain) was added to 5 ml of each PC sample. PCs were incubated with calcium at room temperature (20-22 ºC) for 2 hours to stimulate platelet degranulation. A fibrin clot was formed in each PC sample. Clots were released from the walls of the tubes and centrifuged at 1720 x *g* for 8 minutes. The supernatant plasma was obtained, aliquoted and frozen at -80ºC for later determinations of TGF-β_1 concentration. The time and temperature of incubation was chosen according to the protocol described by Zimmermann et al (20).

Total protein determination-Total protein concentration of all the samples was determined using Warburg's formula (28) by measuring the absorbance of several sample dilutions by Biophotometer (Eppendorf, Barcelona, Spain). The system first takes readings at 260 nm, 280 nm and 320 nm, and the instrument performs a 260/280 ratio, with a 320 nm background correction.

Determination of TGF-β_1 concentration by ELISA- The TGF-β_1 levels of the different PCs and plasma of each horse were analysed. The plasma sample of each horse was obtained by centrifugation of whole citrated blood at 1720 x *g* for 8 minutes. TGF-β_1 concentrations were determined by a sandwich ELISA developed with commercial antibodies for TGF-β_1 (Human TGF-β_1, DY 240, R &D Systems Europe, Abingdon UK). Human TGF-β_1 ELISA kit was used in this study, because of the 99% homology for this peptide between human and equine species (29). In addition, the use of anti-human TGF-beta-1 antibodies for equine TGF-β_1 detection has previously been reported by Charan et al. (30), Carter et al. (10), and, Sutter et al (23). The ELISA was performed in duplicate for each sample. A mouse anti-human TGF-β_1 antibody (R&D Systems (Part 840116) Europe, Abingdon, UK) was used as a capture antibody at a concentration of 2 μg/ml. The detection antibody was a chicken anti human TGFβ_1 (R&D Systems (Part 840117), Europe, Abingdon, UK) at a concentration of 300 ng/ml. The standard straight line was made with recombinant human TGF-β_1. The TGF-β_1 from the samples was activated by a 2.5 N acetic acid/10 M urea solution and the samples were diluted 12 and 24 times. The ELISA was revealed with conjugated streptavidin to peroxidase (R&D Systems Europe, Abingdon, UK) and the colorimetric substratum Fast OPD (Sigma, USA) The reading was performed at 492 nm by an ELISA reader (Anthos, Cutlek, Spain).

Statistical analysis- All the data obtained was analyzed by a commercial statistical software (SPSS Inc, IL, USA) using non-parametric tests, because the different variables were not normally distributed. To establish comparisons between the different variables, PCs were studied with a Kruskall-Wallis test. Post-hoc comparisons were performed using a non-parametric pairwise comparison (Wilcoxon) test. General and specific correlations of the evaluated parameters and between each PC and whole blood citrated samples or plasma were performed with the Spearman rank correlation test (r_s). A $p \leq 0.01$ value was accepted as a statistically significant difference for all the tests.The collection efficiency of platelets and the concentration efficiency of TGF-β_1 for each PC obtained either by the single or double centrifugation tube methods were analyzed using the following formulas (22):

$$\text{Efficiency for platelet collection} = \frac{\text{Platelet count/μl in PC x volume of PC}}{\text{Platelet count in whole blood/μl x volume of whole blood}}$$

$$\text{Efficiency for TGF-}\beta_1\text{ concentration} = \frac{\text{TGF-}\beta_1\text{ levels (ng/ml) in PC x volume of PC}}{\text{TGF-}\beta_1\text{ levels (ng/ml) in whole blood x volume of whole blood}}$$

Results

In general, the processing of PC-A and PC-B (single centrifugation tube method) was shorter (10-12 minutes) than the processing of PC-C and PC-D (double centrifugation tube method (20-24 minutes). After adding the calcium chloride solution, the most important and evident fibrin clot formations were present in PC-C, followed by PC-A, PC-B and PC-D. Fibrin clot formations were normally present 1 hour after PC activation, for this reason each PC remained in incubation with the calcium during 1 additional hour (20).

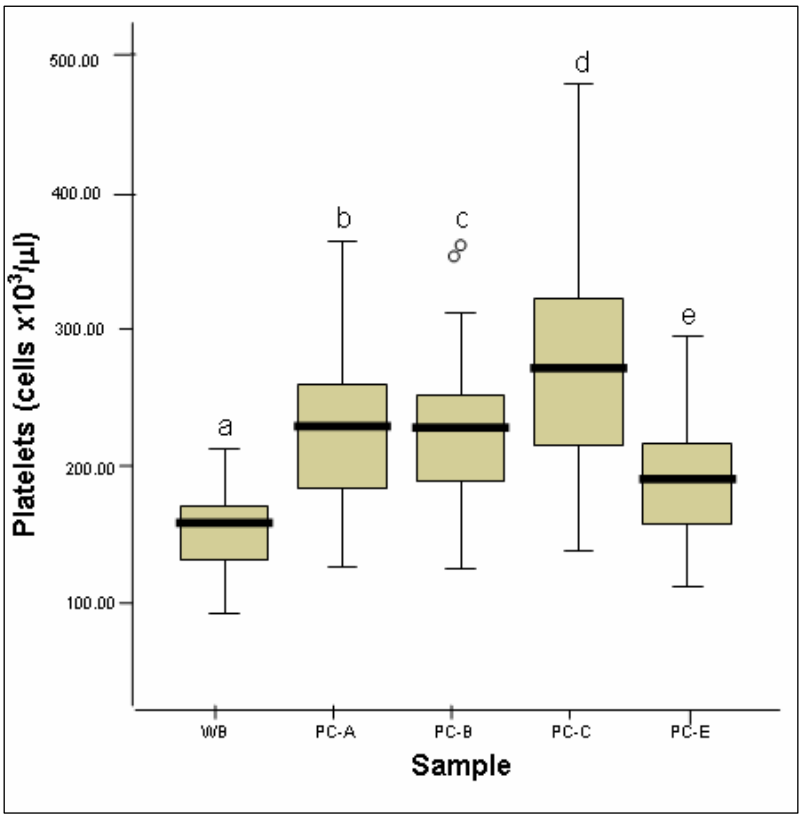

Figure 5.2. Box plots of the median values for platelet cytometric count of whole citrated blood samples and four platelet concentrates obtained by the single and double centrifugation tube methods. [a,b] Different letters represent significant (p<0.01) differences between groups. Whiskers represent the 95% confidence interval. *See* **Table 1** for key.

Packed cell volume, WBC total counts, the relative and absolute numbers of neutrophils, lymphocytes, eosinophils, basophils, and LUCs are presented in **table 5.1**. Values for platelet counts, MPV, PDW, MPC, MPCDW, large platelet counts, platelet clump counts, total protein (mg/ml), and TGF-$\beta_1$levels (either in ng/ml or ng/mg of total protein) for whole blood or plasma and each PC are presented in **table 5.2**.

Hemogram findings- PCV values were significantly (p<0.01) different between the whole blood samples and each PC and also between each PC. Platelet concentrates obtained

either by the single or double centrifugation tube methods had significant (p<0.01) increases in their platelet counts compared with whole blood samples. Platelet counts and leukocyte counts were statistically different (p<0.01) for each PC and PC-C had the highest number in median values (*see* **Table 5.2** and **Fig. 5.2**). Platelet concentrations for PC-A, PC-B, PC-C and PC-D were 45%, 44%, 71% and 21% higher, respectively, compared to the same values for citrated whole blood samples. PC-C had the highest median platelet count, but also had the highest RBC and WBC contamination (*see* **Table 5.1** and **Fig 5.3**). The relative and absolute counts for neutophils, lymphocytes, monocytes eosinophils, and basophils were significantly (p<0.01) different for whole blood samples and each PC. Lymphocytes, monocytes, basophils and LUCs tend to increase in the PC-C samples, but there is a lower proportion of neutrophils and eosinophils than whole blood (*see* **table 5.1**). Large platelet counts were statistically different between the whole blood and each PC and also between each PC. Platelet clumps counts were not statistically different between whole blood and PC-C. However platelet clumps counts were statistically different between each PC. Platelet clumps counts from PC-A, PC-C and PC-D were statistically different from whole blood samples (*see* **Table 5.2**). MPV increased in PC-A and in the PC-B samples, and MPC also significantly decreased in those samples, suggesting a certain degree of platelet activation occurred. (*see* **Table 5.2**).

The PDW values were statistically not different between all the samples. The MPCDW values were statistically not different between whole blood and PC-C, and between PC-A and PC-B and PC-D. However, the MPCDW values were statistically different between whole blood and PC-A, PC-B and PC-D, and between PC-C and PC-A, PC-B and PC-D.

Total protein analysis- Plasma total protein levels were not significantly different between PCs. However, significant (p<0.01) differences for total protein levels were found between PC-B and PC-D. We noticed that PCs tended to have lower total protein concentrations in comparison to whole blood samples although the differences were not statistically significant (*see* **Table 5.2**)

TGF-β_1 analysis- The TGF-β_1 (either ng/ml or ng/mg of total protein) levels were significantly (p<0.01) different between whole blood samples and each PC, except for PC-A. However, this GF levels were statistically identical between PC-B and PC-C. The TGF-β_1 levels in PC-B and PC-C were significantly (p<0.01) higher than for the plasma or other PCs (*see* **Table 5.2**). TGF-β_1 concentrations for PC-A, PC-B, PC-C and PC-D were 38%, 44%, 44% and 37% higher, respectively, compared to citrated whole blood samples.

Statistical correlations- A significantly (p<0.01) positive correlation between the WBC count and the platelet count (r_s= 0.67) was found. When the specific values for each PC and whole blood samples were analyzed, a significantly (p<0.01) positive correlation between

Table 5.1. General results of the variables of the study (Data is presented as the median (range)).

		Single centrifugation tube method		Double centrifugation tube method	
Variables	**Whole blood**	**PC-A**	**PC-B**	**PC-C**	**PC-D**
PCV (%)	26.3 (23-38) a	0.0 (0.0-0.6) b	0.1 (0.0-0.1) c	0.2 (0.0-0.8) d	0.0 (0.0-0.1)e
WBCs (cells x $10^3/\mu l$)	7.1 (5.3-11.2) a	4.1 (7.6-5) b	3.1 (1.8-5.2) c	8.4 (3.3-18.1) d	0.93 (0.62-2.44) e
Neutrophils (cells x $10^3/\mu l$)	3.8 (2.8-6.2) a	1.7 (0.8-4) b	1.1 (0.5-2.5) c	3.3 (0.8-6.9) d	0.21 (0.1-0.6) e
%	58 (38-70) a	37 (23-62) b	32 (20-58).c	36.5 (22-59)c. d	21 (12-46) e
Lymphocytes (cells x $10^3/\mu l$)	2.5 (1.1-4.3) a	2.3 (1-4.5) a	1.9 (0.8-3.8) b	4.8 (2-12) c	0.7 (0.4-2) d
%	34 (20-56) a	57 (34-71) b	63 (39-75) c	57 (38-75) d	76 (51-85) e
Monocytes (cells x $10^3/\mu l$)	0.24 (0.2-0.4) a	0.14 (0.1-0.54) b	0.1 (0.04-0.3) c	0.34 (0.6-1.1) d	0.02 (0.1-0.6) e
%	3.3 (2-6) a	3.4 (2-8) a. b	2.8 (2-7) c	3.95 (1.40) d	2 (0.8-6) e
Eosinophils (cells x $10^3/\mu l$)	0.3 (0.2-0.9) a	0.02 (0-0.6) b	0.1 (0-05) c	0.04 (0.1-0.12) d	0 (0-0.2) e
%	4 (0.4-12) a	0.4 (0-1.7) b, d	0.4 (0-1.6) b, c	0.4 (0-1.5) b, c	0.4 (0-1.5) b, c, d
Basophils (cells x $10^3/\mu l$)	0.02 (0.1-1) a	0.01 (0-0.04) b	0.01 (0-0.3) c	0.2 (0-0.08) a, d	0 (0-01) e
%	0.3 (0.1-1) a	0.2 (0.1-0.8) b	0.2 (0.1-0.7) c	0.2 (0.1-0.7) d	0.2 (0-12) e
LUCs (cells x $10^3/\mu l$)	0.02 (0.01-0.05) a	0.03 (0.01-0.06) b	0.02 (0.01-0.05) b, c	0.07 (0.02-0.2) d	0 (0-0.01) e
%	0.4 (0.1-0.7) a	0.75(0.8-1.2) b	0.7(0.5-1.3) a	0.8(0.08-1.6) c	0.5(0-1.1) d

LUC= Large unstained cell. PC= Platelet concentrate [a,b] Different letters represent significant ($p<0.01$) differences between groups.

the platelet count for whole blood and the platelet counts for each PC was found (PC-A (r_s= 0.85), PCB (r_s= 0.84), PC-C (r_s= 0.76), and PC-D (r_s= 0.83)).

When the TGF-β_1 levels were analyzed, an interestingly significant (p<0.01) negative correlation was found between this GF and the total protein levels (r_s= -0.72) of PC-C.

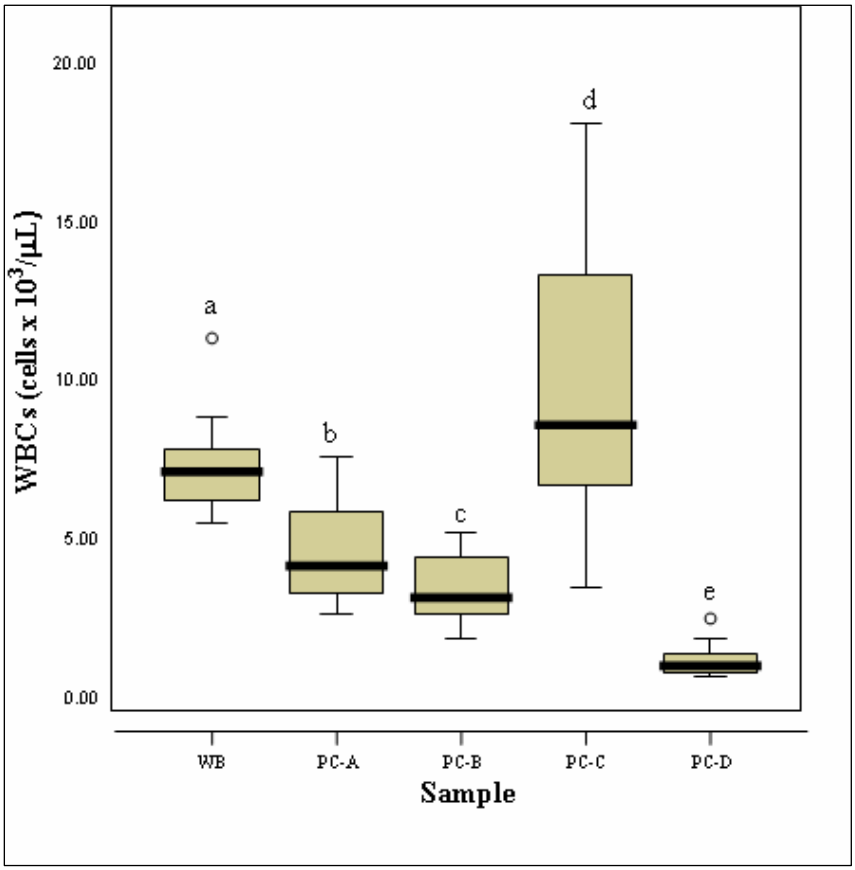

Figure 5.3. Box plots of the median values for WBC cytometric count of whole citrated blood samples and four platelet concentrates obtained by the single and double centrifugation tube methods. [a,b] Different letters represent significant (p<0.01) differences between groups. Whiskers represent the 95% confidence interval. *See* **Table 1** for key.

Platelet counts efficiency and TGF-β_1 concentration efficiency- The platelet count efficiency for PCs obtained by the single centrifugation method was 68% for PC-A and 67% for PC-B. In contrast, the platelet collection efficiency for the PCs obtained by the double centrifugation protocol was 10% for PC-C and 47% for PC-D.

The TGF-β_1 concentration efficiency for PCs obtained by the single centrifugation method was 54 % for PC-A and 58% for PC-B. In contrast, the TGF-β_1 concentration efficiency for the PCs obtained by the double centrifugation protocol was 7.5 % for PC-C and 43% for PC-D.

Discussion

The first tube method protocol for preparing human PCs intended for alveolar-maxillary reconstruction was originally described by Anitua as the PRGF (plasma rich in growth factors) system (12). Although the technique described here seems to be similar to Anitua's technique (12, 15, 22) both procedures differ in certain technical and methodological aspects. Anitua's tube technique (12) presents several technical inconveniences which are related to the risk of bacterial contamination during the preparation of PCs. It is recommended to use a laminar flow chamber and to have trained sanitary personnel to prepare the PCs (12, 15, 22). Although in Anitua's technique a large number of pipettes and other laboratory materials are required (22) in the equine tube technique described here, only plastic syringes and spinal needles are used. In the authors' opinion, the use of these simple materials diminishes costs and time, and facilitates PC preparation but also diminishes the risk of bacterial contamination. The availability of a laminar flow chamber should be always guaranteed when this protocol is used, especially when equine PCs will be used therapeutically (*see* **Sections III** and **IV** of this thesis).

It should be borne in mind that the human tube centrifugation protocol is different from the equine tube technique described here. In our preliminary studies, it was observed that Anitua's centrifugation protocol (450 x *g*/8 min) (22) was not applicable for equine platelets, since the effect of sedimentation on these cytoplasmatic fragments is different (31) from the same effect on human platelets (32). In this study, it could be seen that with single centrifugation (120 x *g*/5 min), the platelets and WBCs (but to a lesser degree) were almost uniformly distributed either in the lower 50% first platelet fraction (PC-A) or in the 50% second supernatant platelet fraction (PC-B) (*see* **Tables 5.1-5.2** and **Figs 5.2-5.3**). With the single tube centrifugation performed here, it was impossible to replicate the results observed with the human tube protocol (15, 22), which concentrates the highest number of platelets between the first two millimetres from the upper line of the PCV and the first 75% of the remaining plasma (22). Anitua calls this portion the 'plasma rich in growth factor' (PRGF) and the remaining 25% of plasma is called 'plasma poor in growth factor' (PPGF) (12, 15, 22). In this sense, equine practitioners should not perform the same human tube protocol to obtain PCs from equine blood. However, the human buffy coat and apheresis protocols can be used for the preparation of equine PC without any modification (10, 23).

Despite the statistical differences that were found between the TGF-β_1 levels of whole blood samples and of each PC (*see* **Table 5.2**), the TGF-β_1 levels were almost uniformly distributed and did not correlate with the platelet or WBC counts for each PC or for the whole blood samples**.** Although the levels of only one GF were analyzed in this study, there are other important growth factors that could be present in high concentrations in PCs whose levels could depend not only on platelet counts but also on the white blood cell population and liver production, amongst other things (20). As occurs with Anitua′s tube technique (22), in the

technique described here a very low quantity of leukocytes are concentrated in comparison with the buffy coat and apheresis methods (equine or human). The real role of the leukocytes present in human or equine PCs prepared for wound healing has never been defined very well, and their true effect is completely unknown because there is lack of reliable research data (20, 22). It should be considered that when equine PC-C was injected intraarticularly in horses with severe joint disease (*see* **Section III** of this thesis), joint effusion and inflammation was observed during the first days of post-treatment. These clinical effects could be explained by the chemotactic effect of the leukocytes present in high concentrations in that PC. However, this clinical effect was not observed in the horses treated with the same PC for soft tissue lesions (*see* **Section IV** of this thesis). These clinical observations may suggest that the cellular components of the equine PC for therapeutic uses should be modified depending on the type of tissue to be treated. In the study described here, it was found that the TGF-β_1 levels were similar for PC-B and PC-C, but as PC-B presented lower leukocyte counts, it is plausible that these two PCs could be used for different therapeutic purposes. Nevertheless, this is merely a hypothesis and further studies should be performed in order to obtain more reliable data.

An interesting finding of this study was the slightly higher number of LUCs in PC-C in comparison with the other PCs and the whole blood samples. Although LUCs can be associated with immature lymphoid or basophilic cells, these cells are also positively correlated with myeloid CD34+ and stem cells (24). However this positively correlation has not been reported in horses. It is plausible that the positive results observed in human and equine clinical cases treated with PCs could be related not only with the GFs present in these substances, but also with the stem cells injected into injured tissues. In this sense, PC-C could be the best therapeutic substance for equine injuries. Nevertheless, the presence of LUCs is correlated with the WBC count and as mentioned earlier, a high number of leucocytes could produce an inflammatory response in certain tissues, such as articular tissue.

Thrombin is the most commonly used protein for human PC activation (11). But for the same purpose, calcium chloride is used instead in Anitua's technique (12, 15, 22). In the technique described here, calcium chloride is also used because it is an inert substance and it is free from side effects such as immunological reactions that are described as a complication of the use of thrombin (6). Nevertheless, additional investigations into the use of thrombin for the activation of equine PCs obtained with the technique described here are needed, because the GF levels could vary depending on the substance used for the activation procedure (20).

The TGF-β_1 levels in the plasma samples in this study were similar to others that have been reported previously in horses (23, 30). We determined the levels of total protein in all of the samples to adjust the TGF-β_1 levels to those of total protein (ng/mg) in order to correct the dilutional effect produced by the sodium citrate solution present in each tube and because of the calcium chloride solution added (10% of additional volume) to each PC for platelet activation.

However, the TGF-β_1 levels (ng/ml) in this study (see table 2) are within the range reported in equine PCs that were obtained using the apheresis method by Carter et al., (7.4 ng/ml) (10) and Sutter et al., (23.6 ng/μl) and using the buffy coat by Sutter et al (15.3 ng/ml) (23).A negative correlation (-0.72) between the TGF-β1 levels and values of total protein in the PC-C was observed. It should be considered that the most developed clots were always present in this PC. The higher concentration of fibrin in the PC-C clots may kidnap more plasmatic proteins than in the other PCs clots. As a result of clot contraction, higher levels of TGF-β_1 were released in PC-C.

Marx (6) postulated that the ideal PC preparation method should be simple, and easy to perform, with the platelets easily separated from the RBC and sequestered in high concentrations without suffering damage so that they can actively secrete their growth factors. Until now, the authors are not aware of the existence of any research related to the potential damage that an equine platelet may suffer and its premature activation during concentration by the different methods used in equine PC preparation for wound healing purposes. In this study, the viability and structural changes of the equine platelets was investigated by assessing the platelet parameters supplied by a flow cytometry hematology system, such as MPV, PDW; MPC and MPCDW. It is known that these parameters, especially MPV and MPC, are correlated with platelet activation in human beings (27) and dogs (25).

In our study, MPV, MPC, and MPCDW values for each PC obtained by single centrifugation were statistically different to values for the same parameters in the PCs obtained by double centrifugation or whole blood samples. It is possible that the platelets of the PCs obtained by the single centrifugation tube method were more activated than the platelets of the second centrifugation PCs. In addition, these parameters were statistically the same in PCs obtained by double centrifugation and in whole blood samples. One possible explanation of this phenomenon could be the fact that the first PCs (PC-A and PC-B) were obtained using a low centrifugation speed and mixed with RBCs (33). However, it seems that when PC-A was centrifuged again at a double centrifugation force and with a low level of RBCs, the MPV, MPC, and MPCDW values of the second centrifugation tended to decrease and were not statistically different from the whole blood samples values.

This could be explained by the sodium citrate having a partial reverse effect on the platelet aggregation process (33, 34). All the values obtained from the additional platelet parameters evaluated in this study may indicate that the manipulation and centrifugation of each PC does not have any major effect on their viability. The platelet collection efficiency and TGF-β_1 concentration efficiency was higher for the PCs obtained by the single centrifugation tube method compared with PCs obtained by the double centrifugation tube method. Nevertheless,

Table 5.2. Platelet related values and levels for total protein and transforming growth factor (Data is presented as the median (range)).

		Single centrifugation tube method		Double centrifugation tube method	
Variables	**Whole blood**	**PC-A**	**PC-B**	**PC-C**	**PC-D**
Platelets (CF x $10^3/\mu l$)	158 (92-274) a	229 (126-364) b	228 (125-361) c	272 (138-479) d	191 (112-295) e
Large Platelets (cells x $10^3/\mu l$)	21 (6-44) a	31 (9-65) b	32 (14-60) c	35 (11-66) d	30 (8-334) c, b, e
Platelet clumps	136 (40-1041) a	86.5 (36-190) b	72.5 (40-148) c	138 (57-339) a	48 (31-738) d
MPV (fL)	12.3 (7.6-15.6) a	13 (10.6-15.2) b	13 (9.1-15.4) b, c	12.2 (8.6-15) a, d	12.6 (8.9-15.2) a, d
PDW (%)	57.2 (14.2-68.2) a	58.1 (18.3-60.6) a	56.5 (16.7-70) a	56.5 (15.2-61.7) a	56.3 (16-60.5) a
MPC (g/dL)	17.4 (14-23) a	16.5 (14-19) b	16.5 (14.2-19.8) b, c	17.5 (14.-21) a, d, e	16.7 (14-19.2) b, d, f
MPCDW (g/dL)	8 (6.8-9.6) a	7.8 (7-17) b	7.8 (7-8.6) b, c	8 (6.7-9) a	7.8 (6.5-8.6) b, d
	Plasma				
Total protein (mg/mL)	66 (56-87) a	56 (42-78) a.	56 (29-80) a, b	57 (46-69) a	57 (38-74) a. c
TGF-β_1 (ng/mL)	8.3 (1.8-16.4) a	9.4 (1.5-17.8) a	10.3 (1.8-17.9) b	10.5 (1.9-17.1) b	9.9 (1.2-16.2) d
TGF-β_1 (ng/mg of TP)	0.012 (0.003-0.025) a	0.017 (0.0023-0.03) a	0.017 (0.003-0.03) b	0.018 (0.003-0.03) b, c	0.017 (0.002-0.023) d

MPC= Mean platelet component concentration. PDW= Platelet distribution width. MPCDW= Mean platelet component distribution width. MPV= Mean platelet volume. TGF-β := Transforming growth factor beta. TP= Total protein. *See* **Table 5.1** for keys

more profound *in vitro* and *in vivo* research must be performed in order to gain more information about the biological and clinical behaviour of PCs in horses.

Conclusion

One part of our initial hypothesis was validated, since WBC and platelets were statistically different between whole blood samples and each PC. However, TGF-β1 levels for PC-C and PC-D were identical. Finally, the PCs obtained by the single centrifugation tube method presented a statistically significant higher level of platelet activation in comparison to whole blood samples and PCs obtained from the double centrifugation tube method. The results of this study let us think that PC-B and PC-C are maybe preferable for the clinical use in equine patients with musculoskeletal pathologies. However, more research is necessary in order to recommend the use of PCs in equine patients.

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VI. CONCLUDING DISCUSSION

CONCLUDING DISCUSSION

Three hypothesis were evaluated in this thesis: a) The intra-articular or perilesional injection of a PC obtained by the double centrifugation tube method can be useful for the treatment of equine patients with musculoskeletal pathologies, including severe arthropathies (OA and OCD), and soft tissue locomotor pathologies (SDFT tendinopathy and SL desmopathy *see* **Chapters III** and **IV**). b) The use of PCs for the treatment of equine musculoskeletal pathologies is safe (*see* **Chapters III** and **IV**). c) The number of PLTs, WBCs, amongst other cells, and the levels of TGF-β_1 should be different from whole blood and the other four platelet concentrates (PCs) (PC-A, PC-B, PC-D, PC-C) obtained during the preparation of the PC (PC-C) used clinically in this thesis (*see* **Chapter V**).

The injection of an autologous PC produced a statistically significant ($p \leq 0.05$) clinical improvement in the evaluated parameters (e.g.: degree of lameness, ultrasonographic joint and soft tissue musculoskeletal lesions score) of the 14 equine patients studied in this thesis. The positive findings observed in these patients are encouraging, but they should be considered as preliminary results. It is obvious that the low number of patients used in each study and the lack of adequate control groups affect the statistical significance credibility of the results. In this thesis, the possible side effects derived from the injection of autologous PCs in the treated patients were evaluated. The results observed after (44 injections) in the clinical studies presented can be considered conclusive and demonstrative of the clinical safety of the evaluated substance. Bearing in mind the pre-stablished clinical (a and b) hypothesis of this thesis, it can be concluded that clinical hypothesis number one (a) was not completely proven and further clinical studies with a control group and an higher number of patients should be performed. On the other hand, hypothesis number 2 (b) was validated since the patients studied did not present any complications derived from the use of the PCs.

The positive clinical results of this thesis were a motivation to perform the study described in chapter V. Interestingly, we found similarly higher transforming growth factor beta-1 (TGF-β_1) levels in two (PC-B and PC-C) of the four equine PCs and in plasma derived from whole blood. However, the number of cells (mainly platelets and leukocytes) for each PC and for whole blood were statistically ($p \leq 0.01$) different. In this sense, hypothesis number 3 (c) of this thesis was partially validated suggests that PC-B and PC-C can also be potentially used for clinical pourposes.

The cellular and molecular evaluation of PCs obtained with the technique decribed in this study represents an important step forward in order to continue with all the research necessary to understand the whole role of these PCs in equine locomotor pathology. For this reason, *in vitro* research using tendon, ligament and cartilage explants and their constitutive cells could be an initial

step to further define the possible mechanisms of action of PCs. To date, our team is conducting a controlled clinical study evaluating the effect of autologuos PC-C on horses with chronic musculoskeletal pathology (Abellanet et al., unpublished data).

Another important research field to explore are the cellular and molecular characteristics of the equine PCs processed with the technique described here. In this thesis, only the levels of transforming growth factor beta-1 (TGF-β_1) were analyzed. It is known that high levels of this peptide are present in PCs, but it is not the only growth factor they contain. Thus, it is necessary to know the proportion of other platelet-associated growth factors such as TGF-β_2, platelet derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor type 1 (IGF-I) and hepatocyte growth factor (HGF).

As a final conclusion the results obtained in this thesis open an encouraging research field in regenerative therapy for chronic locomotor pathology in horses. It is necessary to point out that this potential research will not only be beneficial for horses. The horse can be considered one of the most important animal models for the study of chronic degenerative musculoskeletal pathologies, such as osteoarthritis (OA), tendinopathies and desmopathies, which remain therapeutic challenge in humans.

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