The Effect of Exercise on the Concentration of Platelets in a Platelet Rich Plasma Preparation

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Human Kinetics)

UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January, 2012

Abstract

Traumatic tendon injuries and tendinopathy are common problems in sports medicine

practice. The active population seeks minimally invasive treatments that speed healing time.

New strategies, such as platelet-rich plasma (PRP) therapies, may achieve this. The use of

PRP in sports medicine has been stimulated by the advancing knowledge regarding the role

of growth factors (GF) in tissue repair. GF concentration is thought to increase linearly with

platelet concentration (Eppley et al., 2004). Several studies are emerging with favourable

outcomes in injection of PRP into the area of injury (Kon et al., 2009, Mishra & Pavelko,

2006). Some postulate that the greater the concentration of platelets in a sample the greater

the healing augmentation (Smith, 2009). There is a lack of literature addressing the

clinically practical issue of how to best maximize the platelet-enhanced product drawn from

the patient. The purpose of this study was to evaluate the effect of 2 exercise intensities on

the concentration of platelets in a PRP preparation. The participants exercised on a cycle

ergometer on three occasions. First, a VO_2max test was carried out. The participant then

exercised, on two separate days, for 15 minutes at 50% (moderate exercise) or at 85%

(intense exercise) of their predetermined VO_2max heart rate. Blood was drawn at baseline

and within 3 minutes post exercise. The samples were prepared into a PRP preparation. The

concentration of platelets was analyzed in the PRP. We found a significant increase in the

concentration of platelets in the post-intense exercise PRP samples. No significant increase

was seen in the moderate exercise condition. A significant effect was found for the mean

differences between pre and post in the moderate versus intense exercise groups. These

results indicate that intense exercise is a practical and safe way to increase the concentration

of platelets in a PRP sample.

Preface

Collaborators and co-authors are the following:

- Dr. Don McKenzie produced the research idea and assisted in development of the research protocol. He provided guidance in the writing of the funding application, proposal, ethics application and thesis. He coordinated committee members for the thesis proposal and defense.

- Dr. Sara Forsyth developed the research protocol, conducted the literature review, recruited subjects and collected, analyzed and interpreted data. She wrote all ethics and thesis documents.

- Dr. Jack Taunton assisted with the development of the research idea and protocol.

- Dr. Michael Koehle assisted with the development of the research protocol.

- Dr. Kathryn Serrano assisted with development of the research protocol with respect to blood spinning, handling and quantification.

- Dr. Maria Trache assisted with statistical analysis

No publications have arisen from the work presented in this thesis document.

This study received full board approval from the University of British Columbia Research Ethics Board H10-01915.

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Acknowledgements

- Dr. Don McKenzie, supervisor and committee members, Dr Jack Taunton and Dr. Michael Koehle

- Lianne Dolan assisted with development of the thesis outline.

- Sarah Neil assisted with $V0_2$max testing and data collection.

Chapter 1: Introduction

1.1 Platelet-Rich Plasma

Platelet-rich plasma (PRP) is defined as a volume fraction of the plasma, having a platelet concentration above baseline (Marx,2001). Baseline is defined as the concentration of platelets in whole blood. There is no specific concentration of platelets in PRP that is universally accepted as clinically relevant. Other terms have been applied to the same concept: platelet-enriched plasma, platelet-rich concentrate, autogenous platelet gel and platelet releasant (Redler et al.,2011).

PRP is prepared by centrifuging whole blood, which separates it into layers. This is due to the various densities of the blood components. The red blood cells being the most dense, form the bottom layer and the plasma the top layer. The platelets and white blood cells settle into a small layer in between, called the buffy coat layer. PRP is extracted by pipetting of the layers above the red blood cells. Some PRP is prepared with a double spin method, wherein the whole blood is spun first to separate the red blood cells from the plasma and the second time to concentrate the platelets in the plasma. The second spin results in the formation of a platelet-poor component and a platelet-rich component (Creaney & Hamilton, 2008). Other PRP is prepared with a single spin just separates the red blood cells from the plasma components. This PRP contains white blood cells. There is no agreed upon gravitational force or duration for the centrifugation. No consensus exists on the optimal method of preparation (Everts et al.,2006).

In general, the term PRP has been used to describe various preparations that are rich in platelets. The various preparations are prepared using different protocols and differ in their content of platelets, growth factors and other cell types such as white blood cells. Injection of PRP has been found to be safe (Engebretsen et al.,2010). An important feature of PRP is that it is autologous and thus concerns regarding immunogenic reactions and disease transmission are largely eliminated.

1.2 Platelets

Platelets, or thrombocytes, are small, irregularly shaped clear cell fragments (i.e. cells that do not have a nucleus containing DNA), 2–3 μm in diameter, which are derived from fragmentation of precursor megakaryocytes. The average lifespan of a platelet is normally just 7 to 9 days. They circulate in the blood of mammals and are involved in hemostasis, leading to the formation of blood clots. Platelets are a source of growth factors.

Platelets are produced in bone marrow by budding off from their precursor, megakaryocytes. Production of megakaryocytes is controlled by the hormone, thrombopoietin which is produced by the liver and kidneys. One megakaryocyte can produce 5-10,000 platelets. Approximately 10^{11} platelets are produced each day. Old platelets are destroyed via phagocytosis in the spleen and by Kupffer cells in the liver by a similar process. A reserve of platelets are stored in the spleen and are released when needed by sympathetically-induced splenic contraction. This occurs during exercise Stewart & McKenzie, 2002).

Platelets function in the maintenance of hemostasis through formation of thrombi when damage to the inner lining of blood vessels occurs. Upon damage to this endothelial layer,

specific factors are exposed to the bloodstream leading to activation of platelets. When platelets are activated they clump together and differentially secrete the contents of their granules. Platelets contain three types of granules, dense granules, alpha granules and lysosomal granules. Dense granules, the first to be released upon activation, contain cytokines involved in platelet activation and recruitment. Alpha granules contain growth factors. Some of these proteins are instrumental in tissue healing (Alsousou et al.,2009).

1.3 Growth Factors

Platelets release a multitude of growth factors upon activation. These are substances that are capable of stimulating cellular growth, proliferation and cellular differentiation. They are important in the regulation of a variety of cellular processes. Greater than 30 bioactive proteins have been identified as being released from alpha granules upon platelet activation. Examples of growth factors released by the alpha granules of platelets are platelet-derived growth factor (PDGF), a potent chemotactic agent, and transforming growth factor-beta (TGF beta), which stimulates the deposition of extracellular matrix. Both of these growth factors have been shown to play a significant role in the repair and regeneration of connective tissues (Eppley et al, 2004). Other proteins released by alpha granules include platelet-derived growth factor (PDGF), transforming growth factor(TGF), platelet factor 4 (PF4), interleukin-1 (IL-1), platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF), insulin-like growth factor (IGF), osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin and thrombospondin-1 (Alsousou et al., 2009). These proteins interact with surface receptors

leading to intracellular signaling and to production of proteins essential to regeneration. The various bioactive proteins may target different stem cell types leading to differentiation and synthesis and regeneration. Creaney (2011) commented that trying to pinpoint one growth factor to one particular action is futile due to the complexity of their actions and interactions with each other and specific tissues. The release of these bioactive proteins from the platelets is thought to be the source of enhanced healing with the use of PRP.

The concentration of platelets in a prepared PRP sample is correlates with the whole blood platelet count from which it is derived (Eppley et al., 2004). Additionally, the concentration of growth factors in a PRP sample correlates with the platelet count (Mishra et al., 2009) but this concentration has been found to vary from subject to subject (Eppley et al, 2004). Although, one may simply conclude that the greater the platelet concentration in a PRP sample the greater the tissue healing effect, this has not been consistently reflected in the literature. In an in-vitro study, Choi et al. (2005) showed a stimulatory effect at low PRP concentration and an inhibitory effect of high PRP concentrations on alveolar bone cells. The lack of clear results in this area may be a result of different methods of preparation and thus variable content of the PRP samples.

1.4 Soft Tissue Injury Pathology

Soft tissue injury, specifically, tendinopathy are common problems in sports medicine practice and have limited treatment options. The mainstay of treatment has been physical therapy and non-steroidal anti-inflammatories (NSAIDS). The time course to recovery can

be protracted.   Due to this, the active population seeks treatments that speed healing time and are minimally invasive.

Tendinopathy is a general term for clinical conditions associated with overuse in and around tendons. Tendinosis describes a degenerative pathological condition without a significant inflammatory component (Khan et al., 2000). These terms describe the findings of a failed healing response further described below.

Tendon and ligament injuries can be classified as acute or chronic. Acute injury involves tearing of the collagen fibres with subsequent hematoma formation. The tissue then proceeds through a general healing cascade including inflammation, proliferation and regeneration followed by repair and remodeling. This has been referred to as tendinitis, reflecting the contribution of inflammation.

Chronic tendon injuries are commonly associated with overuse and a failure in the healing cascade.   The pathology includes collagen fibre disruption, mucoid degeneration, and neovascularisation without significant inflammation. Histologically, the tendon appears disordered with haphazard proliferation of tenocytes, intratendinous collagen degeneration, fibre disorientation and thinning, hypercellularity and scattered vascular ingrowth. (Khan et al., 1998). There is a subsequent increase in noncollagenous matrix. These changes result in structural disruption with a decreased ability to absorb load that can make the tendon more susceptible to damage. It is thought that growth factors may be useful in treating chronic tendinopathy due their effects on angiogenesis and collagen synthesis (Molloy et al., 2003).

1.5 Current Treatments for Tendon Injury

The commonly recommended conservative treatments for management of tendinopathy have been anti-inflammatory medication, activity modification and physiotherapy. Some therapies that have be investigated with randomized controlled trials are NSAIDs, eccentric exercises, glyceryl trinitrate patches, ultrasound and shock wave treatment, and various injections (Maffulli et al., 2010B). These are briefly reviewed below.

1.5.1 NSAIDs

NSAIDs are commonly utilized for their analgesic, anti-inflammatory and antipyretic properties. The mechanism of action of NSAIDs is through non-specific inhibition of the enzyme cyclo-oxygenase. This results in blocking the production of prostaglandins from arachidonic acid. Prostaglandin inhibition decreases the inflammatory response. NSAIDs have been used in tendon conditions because of their anti-inflammatory properties. However, it is now widely accepted that tendon injuries do not have a significant inflammatory component (Khan et al., 2000). It is also well documented that there are theoretical concerns with respect to NSAIDs impeding the normal healing cascade. Sovitz and Johnson (2003) note that inflammation is a necessary component of healing. The influx of inflammatory cells and blood remove debris and recruit cytokines to the site of injury. By blocking the inflammatory phase, NSAIDs may delay healing. In addition to blocking the inflammatory cascade, there is theoretical concern that NSAIDs may increase leukotiene production and decrease fibroblast production and thus cause tendon damage (Paoloni & Orchard, 2005)

Andres and Murrel (2008) conducted a literature review looking at NSAIDs in the treatment of tendon conditions. They found 37 randomized clinical trial and systematic reviews. They concluded that NSAIDs appear to provide some short term pain relief, but long term efficacy has not been demonstrated. Further, the long term use of NSAIDs increases the risk of side effects such as gastrointestinal disturbance, altered renal function and hypertension.

1.5.2 Eccentric Exercise

Eccentric exercises have been proposed counteract the failed healing response in chronic tendinopathy by promoting formation of collagen fibre cross-linking within tendon resulting in tendon remodeling. The musculotendinous units will undergo structural adaptation that will protect them from increased stress and thus prevent further injury (Maffulli et al. 2010). Eccentric training programs have been found to be beneficial in the treatment of Achilles tendinopathy (midsubstance greater than insertional), and lateral epicondylitis (Andres & Murrel, 2008). A recent 5-year follow-up study investigating VISA-A score in patients treated with an Alfredson's heel drop exercise program. The investigators also recorded pain status, alternative treatments and ultrasonographic neovascularization. They found that from baseline and one-year the VISA-A score had significantly increased at 5-years with a decrease in tendon thickness. They concluded that the patients could continue to improve, up to 5 years after the completion of the program but mild pain may remain (Van der Plas et al., 2011).

1.5.3   Glyceryl Trinitrate Patches

Glyceryl trinitrate (GTN) patches are a topical application of nitric oxide (the endothelium-derived relaxing factor). The exact mechanism of this intervention is not completely clear but is postulated to exert its effects through augmented blood flow. There have been several randomized controlled trials that provide evidence for the administration of nitric oxide directly over the area of tendinopathy (Andre & Murrell, 2008). In non-insertional Achilles tendinopathy, lateral epicondylitis, and supraspinatus tendinopathy GTN patches were found to decrease pain, increase tendon force, improve functional measures, and improve symptom resolution (Paoloni & Orchard, 2005).

1.5.4   Extracorpeal Shock Wave Therapy

Shock waves are acoustic waves associated with a sudden rise in pressure. The rationale for the use of extracorpeal shock wave therapy (ESWT) is stimulation of the soft-tissue healing with inhibition of pain receptors (Maffulli et al.,2010B). ESWT has been shown to result in increased tenocyte proliferation and increased expression of transforming growth factor. The most convincing evidence for it use is in calcific tendinopathy of the rotator cuff tendons (Andres&Murrell,2008). Other studies indicate results comparable to eccentric training in the treatment of Achilles tendinopathy (Maffulli et al. 2010B). Further investigation is needed to delineate the most effective protocol.

1.5.5 Injections

A number of substances have been introduced for injection in and around tendon injuries. Some examples of these are dextrose, saline, corticosteroids, autologous blood, PRP and polidocanol.

Neovasularization with nerve ingrowth are histological features found in those with tendinopathic tendons. These features are not seen in normal tendon. The new blood vessels and nerves may be a source of pain (Maffulli et al.,2010A).

The collective hypothesis behind these injections is that they produce local mechanical effects resulting in destruction of the neovessels thought to be largely responsible for the pathology. Destruction of these blood vessels would result in obliteration of the accompanying nerves and thus decreasing associated pain.

1.5.5.1 Corticosteroid Injections

Corticosteroid injections have played a major role in the treatment of tendinopathy. Several studies have found good short-term (>6 months) pain control after corticosteroid injection for lateral epicondylitis and shoulder impingement (Coombes et al.,2010). No long-term benefit has been found (Andres & Murrell, 2008). A systematic review found that, for lateral epicondylitis, no intervention was favored in the intermediate and long term when compared to corticosteroid injection (Coombes et al.,2010). As this was a systematic review, number

and location of injections was not specified. There are also concerns regarding the safety of corticosteroid injection in the treatment of tendinopathy. The safety profile is increased when the injections are carried out under fluoroscopic guidance and thus are accurately delivered around the tendon and not within it (Coombes et al., 2010).

1.5.5.2 Sclerosing Injections

Doppler guided injection of the vascular sclerosant, polidocanol, has been evaluated in the management of several tendinopathies. The theory underlying this is to target the pain producing neural ingrowth and neovascularisation. By sclerosing the new vessels it is thought that the pain generating nerve fibres will be eradicated, either directly by destruction, or indirectly, by ischemia. Studies have evaluated the use of sclerotherapy in the management of tennis elbow, patellar tendinopathy and Achilles tendinopathy (Rabago et al., 2009).   The results were favourable in terms of pain relief. Long term benefit has been reported for chronic mid-portion Achilles tendinopathy (Lind et al., 2006) and for patellar tendinopathy (in Andres&Murrell, 2008). Lind et al. (2006) also looked at Achilles tendon structure at 2 years follow up after treatment and found ultrasonographically improved tendon structure.   Less favorable long term benefit was found for lateral epicondylitis (Rabago et al.,2006). In terms of safety profile, pain with injection and post-injection itching and burning have been reported (Coombes et al.,2010).

1.5.5.3 Autologous Blood Injection

Autologous blood injection refers to the re-injection, into the area of injury, of a few milliliters of blood taken from the patient (Creaney & Hamilton, 2008). The theory behind

the injection of autologous blood into tendinopathic areas is similar to that behind the injection of PRP: local application of bioactive proteins that will induce the healing response. In a prospective, double-blind, randomized trial Creaney et al.,(2011) directly compared autologous blood injections to PRP injections in the management of treatment resistant elbow tendinopathy. At 6 months, the authors observed a 66% success rate in the PRP group versus 72% in the autologous blood group. At the 6 month follow-up they found a higher rate of subsequent surgery in the autologous blood group (20%) versus the PRP group (10%).

1.5.5.4   PRP Injection

PRP is defined as a volume of the plasma fraction of autologous blood having a platelet concentration above baseline. (Marx, 2001). It has been investigated in various areas of medicine for it potential to promote healing in tissues. PRP was initially utilized in oral surgery where it has been found to enhance tissue healing (Smith, 2009). PRP has been utilized intraoperatively for healing enhancement during hip, knee and shoulder procedures (Kon et al., 2010). PRP is also used in chronic wound management (Eppley et al., 2004) and is thought to possess antimicrobial properties (Bielecki et al., 2007). PRP has been found to reduce post-operative wound infections and to have in vitro antibacterial activity (Englebretson et al., 2010).

 As reviewed in Section 1.3, the properties assigned to PRP are due to release of bioactive proteins upon platelet activation. The initial release begins within 10 minutes of platelet activation and continues for the remaining life span of the platelet. Theses bioactive proteins include growth factors and cytokines released from the alpha and dense granules of

the platelets.   Simply put, collectively, they activate intracellular signaling pathways that result in production of proteins required for cellular proliferation, matrix formation and collagen synthesis. This has been seen in vitro with the application of PRP. De Mos et al., (2008) cultured human tenocytes in various concentrations of platelets. They found that PRP had a positive effect on collagen production, cellular proliferation and expression of matrix-degrading enzymes and endogenous growth factors. They concluded that use of PRP in tendon injuries may accelerate the degradation of the damaged areas of tendon and promote angiogenesis and formation of the fibrovascular callus.

An in-vitro study on rabbit patellar tendons investigated the effect of increasing doses of PRP-clot releasate (PRCR), which is a combination of growth factors released from PRP clots, on tendons stem cells (TSC) and collagen production (Zhang & Wang, 2010). They found that with increasing dosages of PRCR the tendons stem cells became large, well spread and highly elongated. They found that the treatment also markedly enhanced TSC proliferation, tenocyte-related gene and protein expression and total collagen production. They concluded that the PRCR treatment promoted differentiation of tendon stem cells in active tenocytes exhibiting high proliferation rates and collagen production capability. This study suggests that, clinically, PRP can promote tendon healing by increasing collagen production.

Gosens et al.(2011) conducted a randomized controlled trial on 100 subjects with chronic lateral epicondylitis. At the 2-year follow-up, the PRP group had a greater reduction in pain and a greater increase in function than the corticosteroid injection group. They reported no complications of the PRP injections.

Mishra and Pavelko (2006) compared a single PRP injection to local anesthetic injection control group for the management of lateral epicondylitis. The PRP group showed significant improvements compared to the control group at 8 weeks and greater than 12 months.

A study (Peerbooms et al., 2010) found that injection of PRP into chronic lateral epicondylitis was superior to corticosteroid injection in reducing pain and increasing function. This study included a one year follow up which showed that those receiving the PRP injection continued to improve from the time of injection, whereas the corticosteroid group had only an initial improvement with a subsequent decline.

Sanchez et al., (2007) looked at the effect of application of autologous platelet-rich matrices during Achilles tendon surgery. They found that those treated with the platelet-rich product intraoperatively, recovered their range of motion earlier, had no wound complications and took less time to get back to gentle running and to resume training activities.

De Vos et al., (2011) conducted a double-blind a randomized placebo-controlled trial looking at the effect of PRP, combined with eccentric exercise, on tendon structure and neovacularity in chronic mid-portion Achilles tendinopathy. They found no differences between the placebo (saline and eccentric exercise) and PRP groups at the two year follow-up, in terms of tendon structure or neovascularity (de Jonge et al., 2011).

Kon et al. (2009) looked at the effect of PRP injection into the patellar tendon for jumper's knee. Each tendon received three injections separated by 15 days. They found statistically significant improvements in pain and stiffness scores at a 6 month follow up. They reported no complications.

There is the theoretical risk of induction of fibrosis at the injury site. The local application of growth factors could potentially interfere with the healing cascade (Taylor et al., 2011).

The risk of infection is low, however, as with any injection procedure, sterile precautions must be observed. The risk of transmissible infections or allergic reaction is eliminated as PRP is an autologous product.

There has been one report of altered systemic growth factor levels after a local PRP injection. A fall in serum endothelial growth factor with no change VEGF was found after one local PRP injection. This was a small study with 5 subjects and the serum levels of only two growth factors were measured (Banfi et al., 2006). Further investigation into the systemic effects of local PRP injection is required to clarify this issue.

There are is a lack of consensus on the possibility of local or systemic carcinogenic effects of PRP. As growth factors promote division and proliferation of cells it has been hypothesized that this could occur in mutated cells (Taylor et al.,2011). At this time there is no evidence to support this hypothesis.

1.6 Exercise and Blood Composition

Exercise results in reduction in plasma volume. During exercise there is an increase in blood pressure resulting in a net increase in filtration over reabsorption in the skeletal muscle beds. This leads to a fluid shift from the intravascular compartment to the extravascular compartment, resulting in hemoconcentration and thus an increase in the concentration of platelets and other formed elements in the intravascular compartment.

Various studies have looked at the relationships between blood elements and exercise. An 18-80% increase in platelet number and a small increase in platelet size has been cited after strenuous exercise (85% or greater of maximum heart rate) (Bourey & Santoro, 1988). They postulated that the increase in size of platelets was due to release of platelets from the vascular beds of the spleen, marrow and lungs. More recent animal studies have found that the autonomic nervous system controls oxygen delivery via beta adrenoreceptors in the heart and hemoglobin concentration via alpha-adrenoreceptor-mediated splenic contraction (Stubenitsky et al. 1998).

However, a rise in platelets with maximal exercise has also been observed in splenectomized patients (Ohri et al., 1983). This is likely due to fluids shift outlined above. Chen et al., (1989) looked at platelet count after subjects exercised on cycle ergometer at 75% of their predetermined maximal workload until exhaustion. They found that platelets increased 16-17% after exercise.

1.7 PRP Preparation

The separation of platelets from whole blood is based on the different densities of the blood components when they are subjected to centrifugal force. Platelets are the lightest and smallest blood elements and thus remain suspended in the upper plasma layer during centrifugation.

Gravitational platelet sequestration is currently the most common technique utilized in the preparation of PRP (Alsousou et al., 2009). This utilizes a centrifuge system to separate anticoagulated blood into its layers based on the density of the components. The various

commercially available systems employ different methods to extract the PRP layer (Wroblewski et al., 2010). Other methods are standard cell separation and selective filtration technology or plateletpheresis. Standard cell separators use a continuous-flow bowl with a soft and hard spin. Plateletpharesis technology is based on use of a single use disposable filter designed to concentrate platelets from whole blood (Alsousou et al., 2009).

Castillo et al., (2011) compared three commercially available PRP separation systems, all based on gravitational centrifugation. They found no significant differences in mean platelet concentrations in the prepared PRP from the 3 systems. They found a wide variability in the factor increase from baseline platelet counts (1.06 to 4.1 fold increase from baseline). There was a significant difference among all systems in the concentration of WBCs, PDGF and VEGF. One of the systems concentrated leukocyte-poor product, while the other two a leukocyte-rich product. They found a positive correlation between WBC concentration and VEGF and PDGF concentrations. The investigators concluded that the differences in growth factor concentration may be due to the WBC release of their own growth factors. Controversy exists concerning the leukocyte-rich versus poor PRP. Castillo et al. (2010) note that the presence of leukocytes may play an antimicrobial role in addition to providing enhanced immunomodulatory capabilities. Conversely, they note that the presence of neutrophils may increase local inflammation and thus impede tissue recovery.

1.7 Research Question

1.7.1 Purpose

- To determine if exercise increases the platelet concentration in a platelet rich plasma preparation.

- To determine the effect of exercise intensity on the platelet concentration in a platelet rich plasma preparation.

1.7.2 Hypotheses

- The concentration of platelets in a platelet rich plasma preparation will increase with exercise.

- The concentration of platelets in a platelet rich plasma preparation will increase with exercise intensity.

Chapter 2: Platelets and Tendon

2.1 Introduction

Soft tissue injuries, specifically, tendinopathies are common problems in sports medicine practice and have limited treatment options. The mainstay of treatment has been physical therapy and non-steroidal anti-inflammatories. Injured tissue may have poor healing potential and thus the recovery can be protracted.   The active population seeks treatments that speed healing time and are minimally invasive. New strategies such as platelet-rich therapies may prove to lessen healing time.

Chronic tendinopathy is a result of a failed healing response. Clinically, it can present with pain, swelling and decreased function. The pathology of chronic tendinopathy continues to be further elucidated. The term tendinopathy has been adopted to clarify the lack of contribution of an inflammatory component. Chronic tendinopathy is histologically characterized by disorganized proliferation of tenocytes, disruption of collagen fibers and an increase noncollagenous matrix. There is minimal or an absence of inflammation seen. Commonly, neovascularity is a major histological finding. (Maffuli et al., 2010B) Investigation into a correlation between neovascularization and symptoms has yielded mixed results (Van der Plas et al., 2011).   The authors note that circumstances around the time of the examination, such as activity, could affect the evaluation for neovascularization. Tendinopathic tendons are mechanically less stable due to an increase in matrix remodeling (Paoloni et al., 2011). This lack of structural stability makes them more prone to damage and thus a vicious cycle is initiated. The histopathological findings are thought to be a result

of a chronically overloaded state but similar histological changes are observed when a tendon is unloaded. Thus, unloading seems to induce matrix changes similar to those seen due to mechanical overload. (Cook et al, 2009)

Platelets contain granules that house numerous bioactive proteins. The specific function of each protein and how they interact is yet to be clearly defined. These set the stage for tissue healing as they promote cellular chemotaxis, proliferation, and differentiation, removal of tissue debris, appropriate angiogenesis and the laying down of appropriate matrix (Alsousou et al.,2009). The use of platelet-rich therapies in sports medicine has been stimulated by this advancing knowledge regarding the role of growth factors in tissue repair.

Cell culture studies have demonstrated increased collagen production with tenocyte proliferation (de mos et al., 2008). These researchers cultured human tenocytes for 14 days in various concentrations of PRP or a platelet poor product. They found that both conditions resulted in stimulation of cell proliferation and total collagen but only the PRP resulted in increased expression of the endogenous growth factors.

Zhang & Wang (2010) found that with increasing dose of a platelet-rich product tendon stem cells became large, with enhanced proliferation, gene and protein expression and total collagen expression. These findings indicate that the platelet-rich product induced differentiation of tendon stem cells into activated tenocytes. The findings also imply that the greater concentration of platelets resulted in greater effects.

The evidence indicates that the histological effects of PRP are augmented with greater concentrations of platelets. Exercise is a simple and inexpensive method to increase platelet concentration.

The current study objectives are to determine if exercise increases the platelet concentration in a platelet rich plasma preparation and to determine the affect of exercise intensity on the platelet concentration in a platelet rich plasma preparation.

We hypothesize that exercise will increase platelet concentration and that higher intensity exercise will increase platelets to a greater extent than lower intensity exercise.

2.2 Methods

This study received approval from the University of British Columbia Clinical Research Ethics Board. Each subject was given a detailed description of the study and permitted to participate after giving a written, informed consent.

2.2.1 Subjects

Ten individuals were recruited from the community. To participate in the study the subject had to be female and between the ages of 18 and 35 years of age.

The subjects were recruited through emails to triathlon clubs and university departments and posters in the community.

The exclusion criteria included a medical history of blood disorder, diabetes, rheumatoid arthritis, severe cardiovascular disease, infections, immunodepression, uncontrolled hypertension, cardiac disease, a psychiatric condition, pregnancy or other contraindications to exercise. Individuals who had been on medications within five days of testing that may

have affected blood parameters (anticoagulants, non-steroidal anti-inflammatory drugs, anti-aggregants) were also excluded.

Subjects were asked to avoid strenuous exercise in the 24 hours preceding testing. Caffeine consumption, medications and supplements were recorded.

All subjects who attended visit #1 completed the study.

2.2.2 Study Design

All data collection took place at the Exercise Physiology Lab at the Allan McGavin

Sports Medicine Centre at UBC. Each subject followed the same procedure. Subjects came to the clinic on 3 occasions with each visit separated by at least 24 hours. On the first visit, measures of weight, height were followed by a maximal exercise test. On the subsequent two visits, two different exercise conditions were randomly applied using a random numbers chart. Blood was drawn just prior to and three minutes following exercise.

2.2.3 The Maximal Exercise Test

On the first visit each participant's underwent a maximal exercise test, during which maximum oxygen consumption was measured (VO_2 max). This allowed for the determination of maximum aerobic capacity and heart rate. This graded cycle test to exhaustion was done on an electronically braked cycle ergometer (Quinton Excalibur, Lode Groningen, Netherlands). The gas analyzer was calibrated using standard gases and the pneumotach was calibrated with the 3-L calibration syringe. Expired gases were collected and analyzed with the data recorded in 15 second intervals (SensorMedics Vmax Series 29).

The subjects were seated on the bike with a nose clip and mask on. The test was started at workload of 0 Watts and a constant ramp of 25 Watts per minute was applied until volitional fatigue. Heart rate was monitored using a Polar heart rate monitor (Polar Vantage XL, Kempele, Finland).

Three of four of the following are required to satisfy the criteria for achievement of VO_2 max: volitional fatigue, RER>1.1, plateau in VO_2 with increasing work load, attainment of 90% of predicted maximum heart rate.

2.2.4 Visits #2 and #3

The order of the next two visits was randomly assigned. At both visits a baseline blood sample and an immediate (within 3 minutes post-exercise) blood sample were drawn. Heart rate was again monitored using a Polar heart rate monitor (Polar Vantage XL, Kempele, Finland). At one of the visits, each participant exercised on a stationary bicycle at 85% of their pre determined $V0_2$ max for 15 minutes. This was the intense exercise condition. At the other visit, each participant exercised at 50% of their pre-determined $V0_2$max for 15 minutes. This was the moderate exercise condition. The specific level of exercise was determined by plotting $V0_2$ against heart rate. The calculated value for 50% or 85% was then entered into the linear equation for $V0_2$ and heart rate to obtain the heart rate corresponding to the specific $V0_2$ value (50 or 85% of the $V0_2$max). The 15 minute exercise bout was started when the participant's heart rate came within 5 beats of the predetermined goal heart rate. Each participant was coached to maintain their heart rate within the pre-determined range and they were able to view their heart rate.

2.2.5 Venapuncture

All venapuncture was done by the same researcher using a 21- gauge needle using identical

technique. Three millilitres of venous blood was collected into purple-topped tubes

containing EDTA. The blood was centrifuged at 800rpm (146xg) for 15 minutes at a

temperature of 22 degrees celcius. The PRP layer was aspirated using a pipette and placed in

a separate polypropylene tube for quantification. The plasma was evenly drawn off from the

junction of the buffy coat area to the top of the plasma. This was approximately one

milliliter.

2.2.6 Platelet Quantification

Platelet counting was done using a Bayer ADVIA 120 Analyzer (Bayer Corporation,

Tarrytown, NY) by doing optical platelet counts. Two counts were done on each sample

and then averaged. This method of platelet quantification uses a 2-dimensional analysis

based on the simultaneous measurement of laser light scattered at 2 different angles

(Giacomini et al., 2001).

2.2.7 Data Analysis

Paired t-tests were used to compare within subject means between the pre and post platelet

counts in the two exercise conditions (SPSS Statistics 17.0, IBM Cooperation, Endicott,

New York). A paired t-test was also used to compare the mean differences between the pre

and post exercise platelet counts in the two exercise conditions. Statistical significance was

set at $p < 0.05$.

2.3 Results

2.3.1 Subject characteristics

Anthropomorphic data and aerobic fitness are presented in Table 2.1.

Table 2.1: Subject Characteristics

|	
N	10
Age (years)	23.9+-4.51
Height (cm)	166.5+-6
Weight (kg)	60.35+-6.12
VO_2max (ml kg^{-1}min^{-1})	45.73+-5.3

Values are means +- SD.

Individual subject characteristics are presented in Table A-1 (Appendix A).

2.3.2 Platelet Concentration Pre and Post Intense Exercise

The mean platelet concentration in the PRP post intense exercise (85%) was found to be significantly greater ($P<0.05$) than the mean platelet concentration in the PRP pre intense exercise (Figure 2.1).

The mean change from baseline whole blood to post intense exercise PRP was a 2.64 fold increase in platelet concentration.

Intense exercise (85% of V0$_2$max)
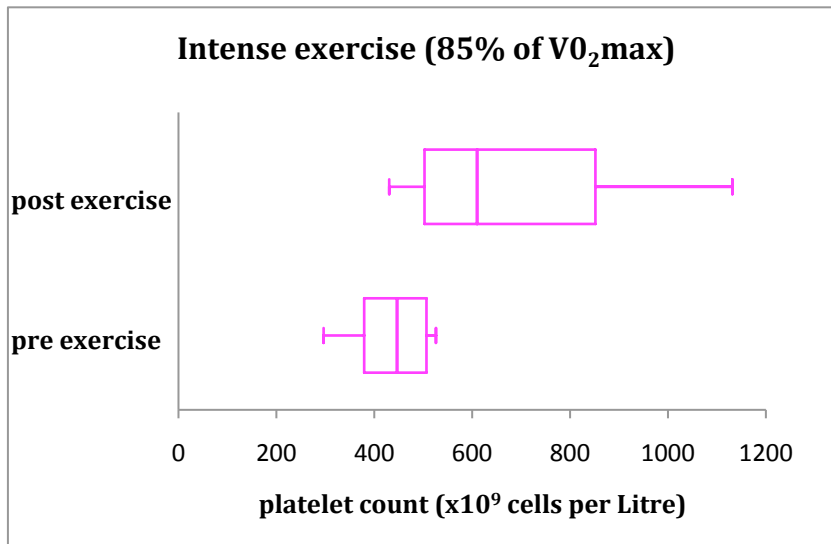
Figure 2.1: Box plot of pre and post exercise platelet concentration for the intense exercise condition. Bars indicate standard deviation.

2.3.3 Platelet Concentration Pre and Post Moderate Exercise

The mean platelet concentration in the PRP post -moderate exercise (50%) was not found to be significantly greater than the mean platelet concentration in the PRP pre- moderate exercise (Figure 2.2).
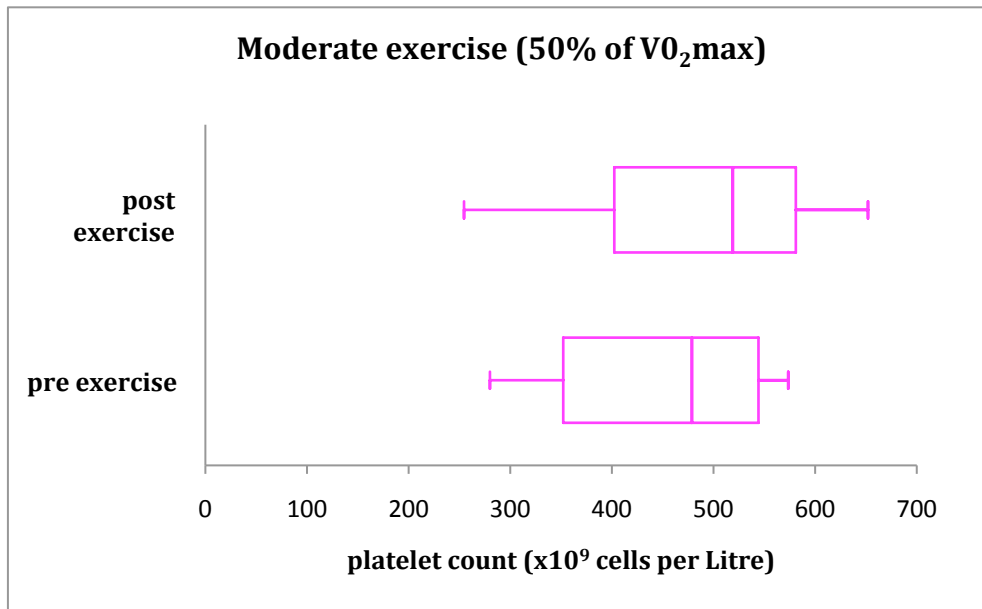
Moderate exercise (50% of V0$_2$max)

platelet count (x10^9 cells per Litre)

Figure 2.2: Box plot of pre and post exercise platelet concentration for the moderate exercise condition. Bars indicate standard deviation.

2.3.4 Comparison of the Differences

The mean difference between the pre and post measures of platelet concentration in the PRP was found to be significantly different (P<0.05) between the two exercise conditions (Figure 2.3).
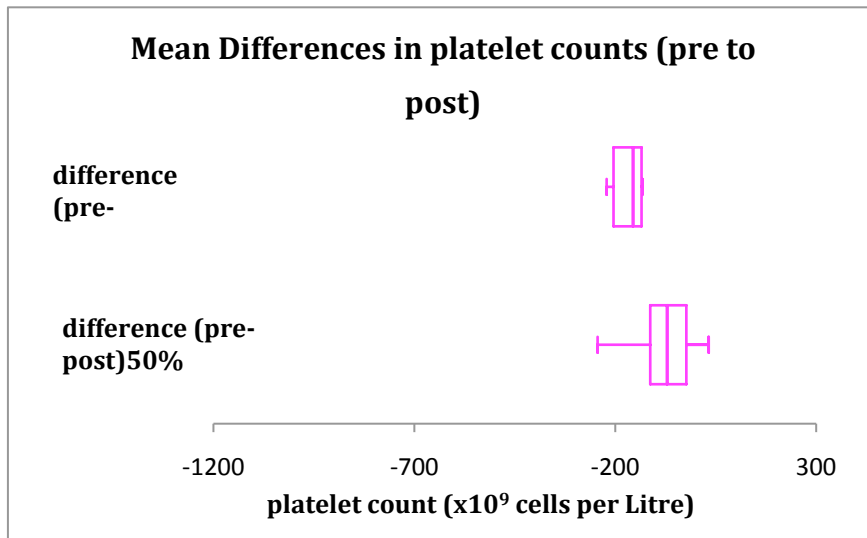
Figure 2.3: Mean differences between pre and post exercise platelet concentrations for the two exercise conditions. Lines indicate standard deviation.
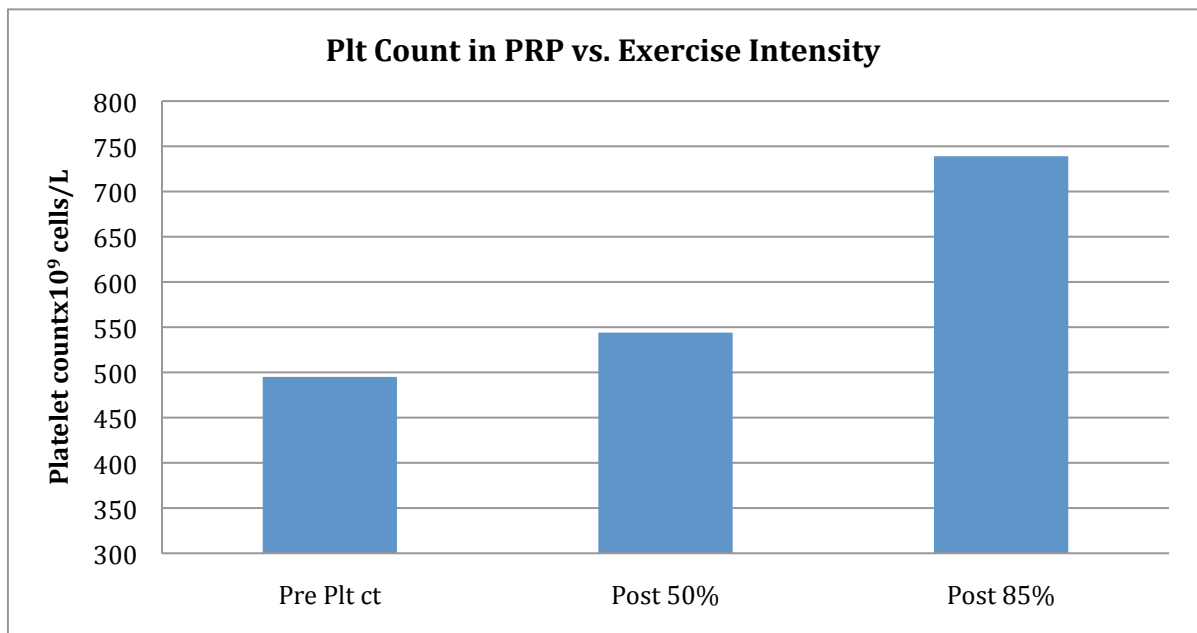
Figure 2.4: Platelet count versus Exercise Intensity

2.3.5 Comparison of Two Methods of Maximum Heart Rate Determination

Additionally, we looked at the difference between the target heart rate determined for the intense exercise condition and compared it to the 85% of maximum heart rate calculated using a common and simple formula: .85(220-age). The difference between the two methods did not reach statistical significance (p=0.67). This was done to illustrate ease clinical applicability. A simple calculation is a practical and according to our data, an accurate way to determine exercise intensity clinically.

Individual data are presented in Table A-2.

2.4 Discussion

The purpose of this study was to examine the effects of exercise on platelet concentration in PRP. Using two different exercise intensities, we found that intense exercise resulted in a significant increase in platelet concentration (1.48x baseline), whereas moderate exercise did not (1.11x baseline). These findings indicate that high intensity exercise is required to result in a statistically significant increase in platelets found in PRP.

It is well accepted that exercise results in fluid shifts within the body. This results in hemoconcentration due to loss of plasma volume, which is due to a fluid shift from the intravascular compartment to the interstitium.   This occurs with the increase in blood pressure and the increase in blood flow to active muscles.   It is not clear as to the intensity of exercise required to result in significant fluid shifts. Wang et al. (1994) concluded that the degree of hemoconcentration induced by acute exercise tended to be related to the

severity of exercise .The results of the current study indicate that 15 minutes of exercise at 50% of the individual`s pre determined maximum was not adequate to a detect significant increase in platelets. The condition of 15 minutes of exercise at 85% of the individual`s predetermined maximum did result in a significant increase in platelets. A key point to further delineate, is what level of exercise results in alteration of hematologic parameters and what are the stimuli for this alteration. The role of fluid shifts and the role of the spleen will be examined.

The role of splenic contribution to altered hematologic profile with exercise was initially based on animal studies (Stubenitsky et al. 1998).   Numerous mammals have the ability to autotransfuse oxygenated red cells from the spleen into circulation during times of stress (Stewart & McKenzie, 2002). This advantage of this mechanism is two-fold; at rest, the spleen of these animals are able to sequester up to 50% of the red cell mass, allowing for reduced blood viscosity and this less work for the heart. Subsequently, during times of stress or heavy work the spleen is able to release this mass, resulting enhanced tissue oxygenation and improved endurance (Stewart et al., 2003). These well studied animal paradigms led to further investigation into the role of the human spleen in exercise.

In humans, the spleen is predominantly a lymphoid organ, and has a minor role in storage with the ability to contain only 200-250ml of blood. This is less than 10% of the total red cell volume. Thus the human spleen was not considered to be a significant contributor to the increase in red blood cells found during exercise. The observation that the human spleen decreases in volume (Allsop et al,, 1992) with exercise led to further interest. Laub et al. (1993) measured erythrocyte content in the human spleen during graded bicycle exercise to maximal working capacity. They found that during increasing exercise the splenic

erythrocyte content decreased linearly and at maximal work load the volume of the spleen was reduced to an average of 34.2% of its original volume. They concluded that splenic autotransfusion contributed to the rise in hematocrit with exercise but was only a partial explanation.

The human spleen contains contractile proteins within the walls of its vessels and capsule, indicating that it has the ability to contract in response to stimuli. It is thought that one of the stimuli is an intensity dependant signal involving the sympathetic nervous system (Stewart & McKenzie, 2002). In a study noted above, Laub et al. (1993) found that the erythrocyte content increased linearly with plasma epinephrine and norepinephrine. The effects of these catecholamines are mediated through adenoreceptors. Increases in red blood cell count have been seen in humans following administration of epinephrine. This effect was not observed in asplenic patients (Stubenitsky et al. 1998).

Allsop et al. (1992) looked at the release of platelets from the spleen during maximal exercise using radio-labeling of red cells, platelets and granulocytes. They found that platelet and leukocytes did not reach their maximal concentration until 5-10 minutes after the end of the exercise. They concluded that platelets and leukocytes took longer to traverse and exit the spleen. This result indicates that contraction is not the primary mechanism by which the spleen releases blood components into peripheral circulation as they reason that if it was all cell types would be released at the same rate. These authors suggest a second mechanism for altered spleen volume being sympathetic directed vasoconstriction of the blood flow to the spleen leading to passive collapse.

The important point in relation to the current study is the intensity of exercise that is required to stimulate the spleen to release platelets into the general circulation. Stewart and McKenzie (2002) looked at spleen volume after 3 bouts of cycling of 5, 10, and 15 minutes at 60% of the subject's pre-determined $V0_2$max. They looked at spleen volume pre and post exercise and found a significant decrease in size of the spleen after each bout and no difference between the different durations. They concluded that the spleen is constantly contracting due to an intensity dependant signal. This conclusion is consistent with the finding (Laub et al., 1993) that increased sympathetic nervous system activity is associated with increased exercise intensity. The intensity dependant signal is likely a catecholamine driven signal. In the current study, the exercise bout at 50% of the subject's pre-determined $V0_2$max was likely insufficient to stimulate the intensity dependant release of blood components from the spleen. This is one possible explanation for the lack of significant difference in platelet concentration between the pre and post exercise samples for this exercise condition.

It is well accepted that during exercise fluid shifts result in hemoconcentration. Pertinent to the current study is the question as to at what intensity and duration do these shifts occur. This information would offer a plausible explanation for the current findings. Novosadova (1977) measured plasma volume after two different exercise conditions (40% and 67% of $V0_2$max). The author found a 13% decrease in plasma volume after maximal effort ($V0_2$max test), a 7% decrease in plasma volume after prolonged submaximal exercise (67% $V0_2$max) and no change in plasma volume after prolonged mild exercise (40% $V0_2$max). It was found that the plasma volume changes were in the first 15 minutes of exercise. These results are interesting in the interpretation of the current study as they offer another plausible

explanation for our findings of significant increase in platelets with 15 minutes of intense exercise (85% VO_2max) and no significant increase with 15 minutes of moderate (50% VO_2max).

In summary, the two key mechanisms involved in increased concentration of platelets and other blood contents during exercise are autotranfusion from the spleen and fluids shifts. Germane to the questions addressed in the current study is the exercise intensity in which these mechanisms commence. The results of the current study indicate that there was no increase in platelets with moderate exercise but there was an increase in platelets with intense exercise. Literature cited above supports plausible mechanisms for these findings.

Although there is no current evidence that has clarified what constitutes a clinically significant concentration of platelets; it is reasonable to postulate that an increase in platelets in a PRP preparation would be clinically beneficial. As reviewed elsewhere, it has been found that there is a linear increase in growth factor concentration with an increase in platelet concentration. Considering this point, a simple maneuver such as exercise, which results in an increase in platelet concentration, would be relevant and beneficial for clinical use.

The results of the current study are generalizable to clinical practice, as exercise is an intervention that is practical and can be easily carried out and measured. We used heart rate to monitor exercise intensity. We compared the heart rate we calculated to correspond to 85% of the individual`s pre determined VO_2max to 85% of the calculated maximum heart rate using the well known formula: max HR=220-age. The difference between the two was

not statistically significant. Again, this illustrates the point that the findings of the current study have clinical applicability.

2.4.1 Summary

The current study illustrates that intense exercise is a safe, simple method to increase platelet concentration in PRP. Moderate exercise did not result in a significant change in platelet concentration.

Additionally, the difference in platelet concentration before and after moderate and intense exercise. Although the difference found in the intense exercise condition did reach statistical significance there is a lack of understanding regarding clinically significant platelet concentrations (Everts et al.,2006). Once clinically significant platelet concentrations are better defined, one could put the current study into a larger context.

Exercise as a maneuver to increase platelet concentration prior to drawing a blood sample is simple. It is possible, however, that an equivalent or greater gain in platelets may be attained by utilizing a particular PRP preparation kit (Castillo et al. 2010).

Another limitation is the use of only 2 exercise conditions varying only in intensity. Perhaps further results may have been found with another condition of prolonged moderate exercise.

2.4.3 Future Directions

Increasing doses of PRP have been shown to enhance cellular processes in cell studies. It would be interesting to pinpoint the platelet concentration at which cellular signal changes

begin to occur. This would demarcate the lowest concentration of platelets that initiates change at a cellular level. From that point, further investigation into what PRP concentration initiates clinically detectable change may be discernable by physical examination, ultrasound or MRI.

There are obvious gaps in preparation and application of PRP. There are various different forms of PRP which need to be universally standardized in some way in order for research to be comparable. These products differ qualitatively and quantitatively. It is difficult to compare results of studies with lack of clarity around the product being injected.

The development of treatment protocols would allow more accurate comparison of studies. This is important as animal studies show that PRP is best combined with mechanical loading to achieve tendon healing (Virchenko et al., 2006). Additionally, the optimal technique and number of injections needs to be delineated.

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Appendix A

Table A-1

Subject	Age (years)	Weight (kg)	Height (cm)	V0$_2$max
1	23	49.5	163.3	49.1
2	32	56.9	170	41.3
3	25	62.4	175	51.0
4	18	56.7	161	48.3
5	25	57.5	172.5	51.5
6	19	60.7	162	39.9
7	26	65.9	167	49.0
8	23	72.4	174.5	37.2
9	19	62.4	161	45.3
10	29	59.1	159.6	43.7

Table A-2

Subject	Pre:50% PRP(x10^9 cells/L)	Post:50% PRP(x10^9 cells/L)	Pre:85% PRP(x10^9 cells/L)	Post:85%: PRP(x10^9 cells/L)
1	373.5	501	296.5	430.5
2	516.5	537	748.5	915.5
3	280	395	448.5	585
4	507	570.5	364.5	509
5	964.5	1208.5	526	1551.5
6	553.5	584.5	448	660.5
7	317.5	424.5	413.5	635
8	450.5	494.5	368	500.5
9	573.5	652	951.5	1131.5
10	345	312.5	445	475
Mean+/-SD	488.5+/-196.4	544+/-264.9	501+/-199.8	739.4+/-359.1

Table A-3

Subject	HR calculated via VO_2max	HR calculated via equation: 85%(220-age)
1	158	167
2	166	160
3	166	165
4	154	172
5	166	166
6	175	171
7	185	165
8	166	167
9	176	171
10	162	173