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Healing Characterization of Surgically Induced Core Lesions of the Equine Superficial Digital Flexor Tendon

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For my family

Contents

I Introduction

Tendinopathies are among the most common musculoskeletal disorders in horses (Thorpe *et al.* 2010). This pathology is one of the major causes of horse wastage in the equine industry (Williams *et al.* 2001). Some tendons are more prone to injury than others, and the superficial digital flexor tendon (SDFT) is one of the most commonly affected structures (Ely *et al.* 2009; Singer *et al.* 2008; Murray *et al.* 2006; Kasashima *et al.* 2004). Tendon lesion distribution varies between the different equestrian disciplines, but in general SDFT lesions affect more often the forelimbs than the hindlimbs (Singer *et al.* 2008; Murray *et al.* 2006; Kasashima *et al.* 2004). After damage, the tendon repairs by forming disorganized scar tissue that is of inferior functional quality (Birch *et al.* 1998; Williams *et al.* 1980) and leads to high re-injury rates (Goodship *et al.* 1994). Many of the currently available treatment modalities cannot significantly reduce this high recurrence rate (O'Meara *et al.* 2010; Gibson *et al.* 1997), urging a continuing quest for more effective therapies.

During the last years, the use of autologous blood products for treatment of tendon and ligament injuries has increased (Mishra *et al.* 2014; Deans *et al.* 2012; Bosch *et al.* 2010; Waselau *et al.* 2008). Platelet concentrates seem to be a promising option, as upon thrombocyte activation more than 300 bioactive cytokines and growth factors (GF) (Transforming Growth Factor-Beta (TGF-ß), Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), etc.) that facilitate cellular communication are released. These growth factors are supposed to act as humoral mediators and biological catalysts, promoting tissue healing and regeneration (Mishra *et al.* 2014; Boswell *et al.* 2012). Moreover, *in vitro* studies using platelet rich plasma (PRP) have demonstrated that this therapy stimulates tendon cell proliferation, differentiation, and maturation

(Mishra *et al.* 2014).

There is a variety of platelet preparations featuring different profiles concerning growth factor concentration, kinetics of growth factor release, inflammatory cytokine expression, platelet and white blood cell counts (McCarrel *et al.* 2012; Sundman *et al.* 2011). These variations are likely to have an impact on clinical outcome, and hence the effects of specific platelet preparations on tendon healing need to be investigated. Autologous Conditioned Plasma ® (ACP, Arthrex Inc.) has been described in the literature as a leukocyte-reduced platelet concentrate (Hessel *et al.* 2014; McCarrel *et al.* 2012; Kissich *et al.* 2012). This blood product has been used in equines and humans for the treatment of tendon and ligament injuries (Deans *et al.* 2012; Rindermann *et al.* 2010). *In vitro* studies have shown that ACP allows concentration of platelets and GFs, while at the same time decreases white blood cell (WBC) counts (McCarrel *et al.* 2012; Kissich *et al.* 2012). Lower WBC counts have been directly correlated to a lower concentration of catabolic enzymes (McCarrel *et al.* 2012; Sundman *et al.* 2011).

It is well known that naturally-occurring lesions present a wide variety of sizes and localizations, and that different animals present different healing capacity. Moreover, in equine medicine the recruitment of large cohorts with tendon pathologies is usually difficult. Therefore, the use of these lesions for the evaluation of the effect of a particular therapy, especially when small cohorts are used, can lead to misinterpretation of the results. Several experimental models of equine SDFT tendinopathy have been developed, aiming at gaining a better understanding of the tendon healing process through the study of the response to artificially created lesions (Cadby *et al.* 2013; Schramme *et al.* 2010; Watkins *et al.* 1985; Williams *et al.* 1984). These models have been also used to determine the effect of different therapeutic approaches on tendon healing (Bosch *et al.* 2010; Dahlgren *et al.* 2002). The majority equine tendinopathy models have not been

validated and present several drawbacks that may affect the interpretation of the treatment effects (Cadby *et al.* 2013). It has been recently claimed that the lesions created using the surgically induced tendinopathy model, reported by Schramme *et al.* (2010), closely resemble characteristic features of naturally-ocurring tendinitis. This model creates standardized, long lasting, moderate size, core lesions in the superficial digital flexor tendon and alters compositional and structural parameters of the tendon, as happens in natural clinical injuries (Cadby *et al.* 2013). The aforementioned characteristics make this model attractive since it allows the comparison of different therapeutical modalities using tendon lesions, which closely resemble natural clinical injuries, with a similar size, in the same localization. The fact that the contralateral limb of the same animal can be used as a control, allows a more accurate comparison since it eliminates of the equation the healing capacity differences presented by different horses.

Tendinopathy models have traditionally been used in forelimb SDFTs since as previously mentioned, they are more often affected by natural lesions. Recently, a quadrilateral equine SDFT lesion model was reported in which lesions were induced in the SDFTs of both fore- and hindlimbs, and then randomly treated using different therapeutic modalities (Crovace *et al.* 2010). If fore- and hindlimb SDFT lesions present a similar healing pattern, the possibility of comparing different treatment modalities in the same animal and the increase number of tendons for analysis, would make this model attractive. On the other hand, this model may not be suitable in cases where the therapies present a potential for systemic effects or migration that might affect the tendon healing in the other limbs. Also, multi-limb models usually raise ethical and animal welfare concerns since the animals cannot efficiently shift their weight away from the painful limbs. Moreover, they are exposed to more tissue injury that can eventually cause more pain and a greater risk of infection. The recent use of multi-limb equine tendinopathy models calls for further investigation to determine if healing of SDFT lesions in fore- and hindlimb is comparable.

The first aim of this study was to evaluate the effects of the ACP on the healing of standardized, surgically induced, SDFT core lesions and to compare the development, as well as the outcome, of standardized, placebo treated, surgically induced SDFT core lesions of fore- and hindlimbs in horses. It was hypothesized that intra-lesional ACP treatments at days 7 and 15 after SDFT lesion induction would improve sonographic, biochemical, biomechanical and histological parameters of the tendons when compared to placebo. Our second objective was to determine if differences on the healing pattern between fore- and hindlimb SDFT lesions would preclude direct comparison between fore- and hindlimbs when using this type of experimental model.

II Research publications in peer-reviewed journals

2.1 Effects of Autologous Conditioned Plasma ® (ACP) on the healing of surgically induced core lesions in equine superficial digital flexor tendon

Publication: Pferdeheilkunde Nov./ Dec. 2014, 30(6) (Accepted)

Roberto J. Estrada [1-2], René van Weeren [3], Chris H.A. van de Lest [3-4], Janneke Boere [3], Magaly Reyes[5], Jean Claude Ionita[6], Manuel Estrada [2], Christoph J. Lischer [1]

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*** See complete publication in appendix 9.1.1**

2.2 Comparison of healing of fore- and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon

Publication: Veterinary and Comparative Orthopaedics and Traumatology. Jul 2014, 27(5), 358-365

Roberto J. Estrada [1-2], René van Weeren [3], Chris H.A. van de Lest [3-4], Janneke Boere [3], Magaly Reyes[5], Jean Claude Ionita[6], Manuel Estrada [2], Christoph J. Lischer [1]

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*** See complete publication in appendix 9.1.2**

III Declaration of the work distribution of the authors in the research publications

3.1 Effects of Autologous Conditioned Plasma ® (ACP) on the healing of surgically induced core lesions in equine superficial digital flexor tendon

Authors: Estrada, R.J., Van Weeren, P.R., Van de Lest, C.H.A., Boere, J., Reyes, M., Ionita, J.C., Estrada, M., Lischer, C.J.

Year: Nov. / Dec. 2014. 30(6), (Accepted)

Journal: Pferdeheilkunde

	R.J. Estrada	P.R. Van Weeren	C.H.A. Van Delest	J. Boere	M. Reyes	J.C. Ionita	M. Estrada	C.J. Lischer
Study Design	42%	25%	-	-	-	3%	-	30%
Study Execution	62%	5%	10%	8%	5%	-	10%	-
Data Collection	87,5%	-	10%	-	2,5%	-	-	-
Data Analysis and Interpretation	55%	17%	8,5%	-	2,5%	-	-	17%
Preparation of the Manuscript	60%	12%	-	4%	1%	8%	-	15%

3.2 Comparison of healing of fore- and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon.

Authors: Estrada, R.J., Van Weeren, P.R., Van de Lest, C.H.A., Boere, J., Reyes, M., Ionita, J.C., Estrada, M., Lischer, C.J.

Year: July 2014. 27(5), 358-365. doi: 10.3415/VCOT-13-11-0136.

Journal: Veterinary and Comparative Orthopaedics and Traumatology

	R.J. Estrada	P.R. Van Weeren	C.H.A. Van Delest	J. Boere	M. Reyes	J.C. Ionita	M. Estrada	C.J. Lischer
Study Design	45%	25%	-	-	-	-	-	30%
Study Execution	67%	5%	10%	8%	5%	-	8%	-
Data Collection	87,5%	-	10%	-	2,5%	-	-	-
Data Analysis and Interpretation	55%	17%	8,5%	-	2,5%	-	-	17%
Preparation of the Manuscript	60%	12%	-	4%	1%	8%	-	15%

IV Discussion

Our results indicate that ACP treatment, in surgically induced core lesions, has a limited influence on tendon healing when compared to saline. In this study, the majority of the ultrasonographic, biochemical, biomechanical and histological parameters showed no significant differences between treatments. Nevertheless, the significant decrease of sulphated glycosaminoglycans (GAGs) in the ACP treated tendons can be interpreted as a possible, but apparently limited, beneficial effect on tendon healing. Moreover, this study showed that there are significant differences on tendon healing between fore- and hindlimb surgically induced core lesions treated with saline during the proliferative phase of healing. Twenty-four weeks after lesion induction, the vascularization score, sulphated GAG, and Hydroxylysylpiridinoline (HP) content were significantly different.

It has been recently claimed that platelet concentrates with low leukocyte content might be a suitable option for improving tissue repair (McCarrel *et al.* 2012; Sundman *et al.* 2011). High leukocyte counts in PRP can induce an increase in the production of catabolic and inflammatory cytokines (McCarrel *et al.* 2012; Sundman *et al.* 2011). The ACP, without anticoagulant, used in this study showed a significant reduction of the WBC counts, but no measurable increase of the platelets. Even though it has been previously demonstrated that this kit is able to concentrate platelets using anti-coagulated blood (Hessel *et al.* 2014, Kissich *et al.* 2012; Mazzocca *et al.* 2012), in our study, using blood without anticoagulant, only 6 out of 16 samples presented a slightly increased concentration of the platelets (24% average increase) when compared to conventional plasma. Nonetheless, Wright stains performed on several smears of ACP samples showed extensive platelets aggregation. Due to the aforementioned findings, we believe that the

lack of anticoagulant in ACP kits caused an important sequestration of the platelets in the aggregates, explaining the relatively low platelet counts in the ACP, as the haematology analyzer will have been unable to count the platelets accurately under these circumstances. This hypothesis is also supported by the fact that the PDGF-BB values were significantly increased in the ACP samples. This finding can only be explained by a concentration of the platelets in the ACP. On the other hand, recent studies suggest that the ACP kit might not be as efficient concentrating platelets as it was previously reported (Hessel *et al.* 2014). This might be an easier explanation for the low platelet counts, but would not explain the increase in PDGF-BB concentration. However, our study cannot be directly compared to Hessel´s since ACD-A was not used in the ACP kits. Interestingly, other studies have used ACD-A in the ACP kits (Kissich *et al.* 2012), but in spite of the usage of anticoagulants, an important variability in the average platelet concentration was seen when compared to the data published by Hessel and others (2014).

This study used ACP without anticoagulant, as it has been shown that GF concentrations are higher in serum than in anti-coagulated blood (Zimmermann *et al.* 2005). Interestingly, the GF concentrations achieved in this study were lower than the previously reported values using anticoagulant citrate dextrose solution A (ACD-A) (Kissich *et al.* 2012). On the other hand, several studies have demonstrated that ethylenediaminetetraacetic acid (EDTA) may alter the determination of the growth factor concentration (Bielohuby *et al.* 2013; Zimmermann *et al.* 2005;). In this study, the ACP samples were stored in EDTA vials to avoid further coagulation. This factor may have affected the accuracy of the determination of the growth factor concentrations. Nevertheless, as conventional plasma and ACP samples were treated identically, there is no doubt about the highly significant increase in PDGF-BB concentration in ACP

samples. Even though there was a significant difference in the PDGF-BB concentration, these results were significantly lower when compared to previously reported values (Hessel *et al.* 2014, Kissich *et al.* 2012). Interestingly, a recent study suggests that ACP is apparently not as efficient concentrating PDGF-BB and TGF-β as it was previously reported (Hessel *et al.* 2014), this might suggest that the growth factor differences found in these 3 studies could have been caused by a low repeatability of the ACP kit when using horse blood. This is especially true when comparing the values reported by Hessel and others (2014) and Kissich and other (2012), where ACD-A and a very similar processing protocol were used.

Tendons are multi-unit hierarchical structures mainly composed of collagen molecules (Silver *et al.* 1983). The intra- and intermolecular crosslinks are in part responsible for the physical properties of the tendinous collagen (Tsuzaki *et al.* 1993). HP is one of the major non-reducible collagen crosslinks found in the tendon (Parry 1998). After tendon injury, the newly formed collagen within the lesion is less cross-linked than in normal tendons (Eyre *et al.* 1984). During the remodeling phase, more stable crosslinks are formed resulting in repaired tissue with higher tensile strength and stiffness (Williams *et al.* 1980). GAGs are polysaccharides that are usually covalently attached to core protein, forming macromolecules known as proteoglycans (Esko *et al.* 1999). In tendons, they are in part involved in the regulation of the collagen fibrillogenesis, affecting fibril size and formation rate (Cribb and Scott 1995), and contribute to the biomechanical and structural properties of the extracellular matrix (Parkinson *et al.* 2011). The role of GAG accumulation after tendon damage is seen controversially in the literature. Previous studies in humans and equines indicate that degenerated tendon regions present higher concentrations of sulphated GAGs (Parkinson *et al.* 2010; Birch *et al.* 1998). Synthesis and accumulation of these molecules is usually correlated to an increased cellular metabolism within

injured areas in the tendon (Parkinson *et al.* 2010), and a marked increase of small collagen fibers that usually decreases the mechanical properties of tendons and ligaments (Halper *et al.* 2006). Recently, SDFT injuries treated with stem cells presented a normalization of several key parameters. The significant decrease of the GAG content demonstrated in this study was seen as positive effect of this therapy (Smith *et al.* 2013). On the other hand, a recent study using blood products for the treatment surgically induced core lesions interpreted the increase of intralesional sulphated GAG concentration as a positive feature of tendon healing (Bosch *et al.* 2010).

Even though there is not a clear consensus in the literature, we believe that the fact that the ACP treated tendons showed significantly less sulphated GAGs than the controls, suggests that the therapy might have positively affected the intra-tendinous cellular metabolism. This may thus have a beneficial effect on the characteristics of both the non-collagenous and collagenous matrix of the tendons. The difference in the GAG content may also be interpreted as a faster tendency towards normalization of this parameter when compared to control treated SDFTs. Nonetheless, this interpretation might be daring, without a direct comparison of the GAG content between injured and healthy tendon sections and without having any other significant difference in the rest of the biochemical parameters. The biomechanical tests performed in this study could not demonstrate a significant difference between the mechanical properties of the ACP and saline treated tendons. Therefore, it is most likely that the significant difference in GAG content found in this study does not play a major role affecting the biomechanical characteristics of the tendons. Moreover, 24 weeks after lesion induction, the hindlimb SDFTs showed a significantly higher concentration of HP and significantly lower concentration of sulphated GAGs when compared to the forelimbs. The HP concentration in the hindlimb lesions was close to the values previously reported for normal SDFTs (Lin *et al.* 2005). Nevertheless, the sulphated GAG content was

higher when compared to the same values (Lin *et al.* 2005). Together, the aforementioned findings suggest an ongoing healing process in both fore- and hindlimb SDFTs. The less cross-linked tissue with higher concentration of sulphated GAGs found in the forelimbs might be correlated to a relatively more immature reparative tissue.

As in other tissues, tendon lesions present an increased blood flow after acute injury, allowing cell recruitment and providing humoral mediators, GF and nutrients needed for adequate lesion healing (Yang *et al.* 2012). There is a general consensus that normal equine tendons present no detectable intra-tendinous blood flow when evaluated with color Doppler ultrasound (CDU) (Bosch *et al.* 2011b; Kristoffersen *et al.* 2005). Persistent vascularization in injured tendons has been interpreted as a sign of inadequate healing and incomplete repair (Bosch *et al.* 2011b; Kristoffersen *et al.* 2005; Murata *et al.* 2012). Recent preliminary studies in horses suggest that CDU could be a useful method for the differentiation between a more fragile vascular granulation tissue and hypovascular scar tissues during the remodeling phase of healing (Murata *et al.* 2012). Twenty-four weeks after lesion induction, the forelimb SDFT lesions showed a significantly higher CDU vascularization score. Hence the lower vascular density presented by the hindlimb SDFTs, where 5 out of 8 horses showed no visible vascularization, can be interpreted as a positive step towards normalization of this parameter when compared to the forelimbs.

Interestingly, the significant differences in intra-tendinous vascularization, GAG and HP content were not correlated to significant differences in the ultimate tensile strength and elastic modulus when comparing fore- and hindlimbs. We believe that even though there were healing differences between fore- and hindlimb SDFTs, the overall biomechanical characteristics of the tendons were not affected since other important biochemical parameters didn´t differ significantly.

Both ACP and placebo treated tendons showed a higher stress at failure (σ_{Max}) and a lower elastic modulus when compared to similar models (Bosch *et al.* 2010). Apart from possible differences in the testing equipment, this may have to do with sample handling. Freezing murine tendon samples at -80°C resulted in fibril rupture at higher stresses (Goh *et al.* 2010). The fact that the samples were transported at -150°C degrees might have affected the biomechanical properties of the tendons in this study. However, this factor was similar for all samples and therefore the non-significant difference found between ACP and placebo is most likely to be real.

As previously reported by Cadby *et al.* (2013), the commercial Masson´s Trichrome used in this study stained the normal collagen fibers red, whereas the disorganized reparative tissue was stained blue. The pattern was consistent in all samples, allowing to make a clear distinction between blue and red areas. This staining technique, in conjunction with the imaging analysis system, proved very helpful for the objective quantitative global assessment of the tendon structure.

It has been suggested that leukocyte-reduced platelet concentrates might be superior than PRP stimulating tendon healing, since persistent inflammation incited by the WBC may result in scar tissue formation (McCarrel *et al.* 2012). Comparing our results to similar models (Bosch *et al.* 2011b; Bosch *et al.* 2010), it seems that PRP (non-activated, leukocyte-enriched) has a more profound effect on tendon healing than ACP. On the other hand, even though PRP showed several positive effects on tendon healing, it is still questionable if the chronically increased intra-tendinous vascularization, GAG content and cellularity found 23 weeks post-treatment can be interpreted as beneficial since these features have been previously correlated to poor tendon repair (Smith *et al.* 2013; Birch *et al.* 1998). Therefore, it is of paramount importance to continue

studying the *in vivo* effect of the different platelet concentrate preparations, to determine which is more efficient normalizing biomechanical, biochemical and histological parameters towards those levels found in healthy tendons.

Equine multi-limb tendinopathy models have been recently introduce as an option to compare the effect of different therapeutical modalities between the different limbs of the same animal (Crovace *et al.* 2010). Nonetheless, it is not clear if the healing of fore- and hindlimb tendon lesions behaves similarly. In the case that fore- and hindlimb SDFT lesions present a similar healing pattern, the possibility of comparing different treatment modalities in the same animal, the eventual decrease of animals needed, and the increase number of tendons for analysis, would make this model attractive. However, for studies investigating therapies that have the potential for crossover effects from one limb to another due to systemic effects or cell migration, this multi-limb model may not be a suitable choice. Critiques of this multi-limb approach have also raised concerns over animal welfare issues based on the potential for horses to have increased risk of infection, increased pain and/or the inability to shift their weight off an injured limb. While these issues are certainly of significant concern and were taken into consideration when developing this model, we found that with the management used in this study the horses didn´t present signs of infection or overt pain, neither directly after lesion induction nor throughout the study. Nonetheless, one can argue that pain was subjectively assessed and also pain in all four limbs would preclude them to exhibit signs of discomfort in a particular limb.

The intra-lesional injection of the saline might have influenced the natural healing process, causing more local inflammation and fiber disruption due to distention of the lesion. This might have affected the tendon healing and therefore the final properties of the injured tissues.

However, nowadays, it is common practice to inject different therapeutic modalities into the SDFT core lesions and therefore these results may reflect modern practice better than a lesion that is left untreated. Moreover, all tendons were treated in the same fashion, so the effect would affect lesions in fore and hindlimbs equally.

There are limitations to this study. Even though this surgical tendinopathy model successfully creates lesions emulating the features of naturally-occurring SDFT core lesions (Cadby *et al.* 2013), the fiber disruption caused by the synovial resector does not exactly replicated the degenerative process that is believed to precede natural clinical injuries (Birch *et al.* 1998). A period of 24 weeks is certainly not long enough to evaluate the end stage tendon healing since the final maturation of tendon repair takes approximately one year (Silver *et al.* 1983). Histological evaluations of tendon healing have been traditionally performed using semi-quantitative scores (Bosch *et al.* 2010). An image-processing package was used to objectively analyze the histological sections in this study. The fact that only this technique was used might have caused a loss of valuable information. Even though normal biochemical parameters of the SDFT have been already published (Lin *et al.* 2005), processing healthy sites of the tendons would have allowed a more accurate comparison between the lesion site and the normal tissues.

In conclusion, our study indicates that 2 intra-tendinous ACP treatments, without anticoagulant, during the proliferative phase of healing, in surgically induced SDFT core lesions, have a limited effect on tendon healing when comparing ultrasonographic, biochemical, biomechanical and histological parameters with the control treatment. Nevertheless, the significant decrease of sulphated GAGs in the ACP treated tendons can be interpreted as a possible, but apparently limited, beneficial effect on tendon healing. Long-term placebo controlled clinical trials with

more horses are warranted to determine if this effect is clinically significant. Moreover, our results suggest that fore- and hindlimb SDFT surgically induced lesions exhibit significant differences in several important parameters of tendon healing 24 weeks post-surgery. These differences create significant challenges in using all 4 limbs and accurately interpreting the results that one might generate. Therefore these findings do not support the use of four limb models for study of tendon injury, until the reasons for these differences are much better understood.

V Summary

Healing Characterization of Surgically Induced Core Lesions of the Equine Superficial Digital Flexor Tendon

Roberto J. Estrada, DVM

Tendon pathologies are among the most common musculoskeletal disorders in horses. Some tendons are more prone to injury than others, and the superficial digital flexor tendon (SDFT) is one of the most commonly affected structures. After damage the tendon repairs by forming disorganized scar tissue that is of inferior functional quality, leading to high re-injury rates. Many of the currently available treatment modalities cannot significantly reduce this high recurrence rate, urging a continuing quest for improved therapies. Autologous Conditioned Plasma ® (ACP) has been described in the literature as a leukocyte-reduced platelet concentrate. This blood product has been used in equines and humans for the treatment of tendon and ligament injuries. It has been recently claimed that this preparation might be superior improving tendon healing than other platelet concentrates. However, the effect of this therapeutical approach on tendon healing is unknown. Therefore the first objective of this study was to evaluate the effects of intra-lesional treatment of tendon injuries with ACP. Additionally, even though equine multi-limb tendinopathy models have been previously reported, it is unknown if fore- and hindlimb tendon healing behave similarly. Therefore, the second aim of this study was to compare the healing process of surgically induced, placebo treated, SDFT core lesions of fore- and hindlimbs in horses.

Tendon core lesions were surgically induced in the SDFT of both fore- and hindlimbs in eight healthy horses. At days 7 and 15 after lesion induction, one randomly assigned fore- and hindlimb was treated with ACP and the contralateral one with placebo. Tendon healing was

monitored clinically and using plain and color Doppler ultrasonography (CDU). After 24 weeks, the tendons were harvested for the evaluation of biochemical, biomechanical and histological parameters.

Twenty-three weeks after treatment, the ACP treated tendons presented a significantly lower concentration of glycosaminoglycans (GAGs) (p=0.05) when compared to placebo. Twenty-four weeks post-surgery, forelimb SDFT lesions presented a significantly higher CDU vascularization score (p=0.02) and GAGs concentration (p=0.04) and a significantly lower hydroxylysylpyridinoline (HP) content (p=0.03) when compared to the hindlimbs.

In conclusion, our results indicate that 2 intra-tendinous ACP treatments, without anticoagulant, during the proliferative phase of healing, in surgically induced tendon core lesions, have a limited effect on tendon healing, when comparing ultrasonographic, biochemical, biomechanical and histological parameters with the control treatment. Nevertheless, the significant decrease of sulphated GAGs in the ACP treated tendons can be interpreted as a possible, but apparently limited, beneficial effect on tendon healing. Long-term placebo controlled clinical trials with a more horses are warranted to determine if this effect is clinically significant. Moreover, our results suggest that fore- and hindlimb SDFT surgically induced lesions exhibit significant differences in several important parameters of tendon healing 24 weeks post-surgery. These differences create significant challenges in using all 4 limbs and accurately interpreting the results that one might generate. Therefore these findings do not support the use of four limb models for study of tendon injury, until the reasons for these differences are much better understood.

VI Zusammenfassung

Charakterisierung der Heilung von chirurgischen induzierten „Core Lesions“ der oberflächlichen Beugesehne des Pferdes

Roberto J. Estrada, DVM

Die Verletzung der oberflächlichen Beugesehne, ist eine häufige Erkrankung des Bewegungsapparates beim Pferd. Nach einer Läsion werden die Sehnen in der Regel durch unorganisierte Bildung von Narbengewebe repariert. Die reparierten Strukturen zeigen eine reduzierte funktionale Qualität, was zu einer hohen Wiederverletzungsrate führt. Viele der derzeit verfügbaren Behandlungsmethoden können diese hohe Rezidivrate nicht reduzieren, was weiterhin der Suche nach verbesserten Therapien vorantreibt. Leukozytenreduzierte Thrombozytenkonzentrate wurden zur Behandlung von Sehnen- und Bänderverletzungen bei Pferd und Mensch verwendet. Jüngste Studien haben behauptet, dass diese Therapieform die Sehnenheilung effektiver als andere Thrombozytenkonzentrate fördern könnte. Allerdings ist die Wirkung dieses Therapieansatzes auf die Sehnenheilung unbekannt. Daher war das erste Ziel dieser Studie, die Auswirkungen der intraläsionalen Behandlung von Sehnenverletzungen mit Autologous Conditioned Plasma ® (ACP) zu bewerten. Das zweite Ziel dieser Studie war es den Heilungsprozess von chirurgisch induzierten, Placebo behandelten „Core Lesions“ der oberflächlichen Beugesehne (OBS) zwischen Vorder- und Hintergliedmaße zu vergleichen.

Bei acht gesunden Pferden wurden in der OBS beider Vorder- und Hintergliedmaßen, standarisierte chirurgische Läsionen gesetzt. An den Tagen 7 und 15 nach Induktion der Läsion wurde an je einer Vorder- und Hintergliedmaße unter Ultraschalkontrolle ACP intraläsional

injiziert. Die jeweilige kontralaterale Gliedmaße wurde mit steriler Kochsalzlösung behandelt. Der Verlauf der Sehnenheilung wurde klinisch und mit Graustufen- und Farbdopplersonographie kontrolliert. Nach 24 Wochen wurden die Pferde euthanasiert und die Sehnenproben für die biochemischen, biomechanischen und histologischen Untersuchung entnommen.

Dreiundzwanzig Wochen nach der Behandlung präsentierten die ACP behandelten Sehnen eine deutlich geringere Konzentration von Glykosaminoglykanen (GAG) ($p = 0.05$) im Vergleich zur Placebogruppe. Vierundzwanzig Wochen post-OP zeigten die OBS-Läsionen der Vordergliedmaßen einen signifikant höheren Farbdoppler Vaskularisierungs Score ($p = 0.02$), eine signifikant höhere Konzentration von GAG ($p = 0.04$) und einen signifikant niedrigeren Hydroxylysylpyridinoline (HP) Gehalt ($p=0.03$). Bei der Sehnenheilung von chirurgisch induzierten „Core Lesions" der OBS könnten nach zweimaliger intraläsionalen Behandlung mit ACP, während der Proliferationsphase, wenige signifikante Unterschiede festgestellt werden, wenn ultrasonographische, biochemische, biomechanische und histologische Parameter mit der Placebogruppe verglichen wurden. Die signifikante Verringerung des GAG-Gehaltes bei ACP-behandelten OBS, spricht für einen positiven, aber wahrscheinlich nur geringen, Effekt in der Sehnenheilung. Langfristige Placebo-kontrollierte klinische Studien mit einer höheren Anzahl von Pferde sind notwendig, um diesen Effekt zu bestätigen. Unsere Ergebnisse zeigen auch, dass es Unterschiede bei wichtigen Parametern der Sehnenheilung in den ersten 24 Wochen nach Defektsetzung gibt, wenn die OBS der Vorder- und Hintergliedmaße miteinander verglichen sind. Diese Unterschiede beeinträchtigen die Interpretation der Ergebnisse erheblich, wenn alle vier Gliedmaßen verwendet werden. Unsere Ergebnisse können die Verwendung eines Viergliedmaßen-Sehnenmodells zur Erforschung des Heilungsverlaufes von Sehnenverletzungen nicht empfehlen, solange die Gründe für diese Unterschiede nicht geklärt sind.

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VIII List of abbreviations

AAEP	American Association of Equine Practitioners
ACD-A	Anticoagulant Dextrose Solution A
ACP	Autologous Conditioned Plasma
b.i.d.	bis in die (Latin for "twice daily")
BW / b.w.t.	Body Weight
CDU	Color Doppler Ultrasound
DMMB	Dimethylmethylene Blue
DNA	Desoxiribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	et alii (Latin for "and others")
ES	Elastic Modulus
Fmax	Force at Failure
GAG	Glycosaminoglycans
GF	Growth Factor
HCl	Hydrochloric Acid
HP	Hydroxilysylpyrodinoline
Hyp	Hydroxyproline
IM / i.m.	Intramuscular
IU	International units
IV / i.v.	Intravenous
kg	Kilogram
KHz	Kilohertz

L	Liter
LP	Lysylpyridinoline
mg	Milligram
MHz	Megahertz
ml	Milliliter
mm	Millimeter
MS	Mass Spectrometry
o.p.d.	Once Per Day
PO / p.o.	per os (Latin for oral)
PDGF	Platelet Derived Growth Factor
PDGF-BB	Double B Chain Platelet Derived Growth Factor
PRF	Pulse Repetition Frecuency
PRP	Platelet Rich Plasma
SD	Standard Deviation
SDFT	Superficial Digital Flexor Tendon
T- ES	Total Echoscore
T- FAS	Total Fiber Alignment Score
TGF- ß	Transforming Growth Factor Beta
TL-%	Total Lesion Percentage
TL-CSA	Total Lesion Cross-Sectional Area
TT-CSA	Total Tendon Cross-Sectional Area
ug	Micrograms
umol	Micromole
UTS	Ultimate Tensile Strength
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells

IX Appendix

9.1 Publications

9.1.1. Effects of Autologous Conditioned Plasma ® (ACP) on the healing of surgically induced core lesions in equine superficial digital flexor tendon

Pferdeheilkunde 30 (2014) 6 (November/Dezember) **XXX-XXX**

Effects of Autologous Conditioned Plasma® (ACP) on the healing of surgically induced core lesions in equine superficial digital flexor tendon

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Summary: Tendon pathologies are among the most common musculoskeletal disorders in horses. After damage the tendon repairs by forming disorganized scar tissue that is of inferior functional quality than normal tendon, leading to high re-injury rates. Many of the currently available treatment modalities cannot significantly reduce this high recurrence rate. Autologous Conditioned Plasma (ACP, Arthrex Inc., USA) has been described in the literature as a leukocyte-reduced platelet concentrate. This blood product has been used in equine and human medicine for the treatment of tendon and ligament injuries. However, the effect of this therapeutical approach on tendon healing is unknown. Core lesions were surgically induced in the Superficial Digital Flexor Tendons (SDFT) of both fore- and hindlimbs in eight healthy horses. At days 7 and 15 after lesion induction one randomly assigned fore- and hindlimb was treated with ACP and the contralateral one with saline. This study used data from the forelimbs SDFTs only. Gray-scale and color Doppler ultrasonographic parameters monitored throughout the study didn t differ significantly at any time point. Twenty-two weeks after the last treatment, the ACP treated tendons presented a significantly lower concentration of sulphated glycosaminoglycans (GAGs) ($p \leq 0.05$) when compared to saline. Other compositional, biomechanical and histological parameters presented no significant differences. Our study indicates that 2 intratendinous ACP treatments (without anticoagulant) during the proliferative phase of healing in surgically induced tendon core lesions, have a limited effect on tendon healing when comparing ultrasonographic, biochemical, biomechanical and histological parameters with the control treatment. Long-term placebo controlled clinical trials with more horses are warranted to determine if this effect is clinically significant.

Keywords: horse / platelet concentrate / Autologous Conditioned Plasma (ACP) / tendon / healing / model / orthopedics

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Auswirkungen von Autologous Conditioned Plasma® (ACP) in der Heilung von chirurgischen induzierten Core Lesions der oberflächlichen Beugesehne des Pferdes

Sehnenerkrankungen gehören zu den häufigsten Erkrankungen des Bewegungsapparates beim Pferd. Nach einer Läsion werden die Sehnen in der Regel durch unorganisierte Bildung von Narbengewebe repariert. Die reparierten Strukturen zeigen eine niedrige funktionale Qualität, was zu einer hohen Wiederverletzungsrate führt. Viele der derzeit verfügbaren Behandlungsmethoden können diese hohe Rezidivrate nicht reduzieren, was eine Fortsetzung der Suche nach verbesserten Therapien vorrantreibt. Autologous Conditioned Plasma® (ACP, Arthrex Inc., USA) ist in der Literatur als ein leukozytenreduziertes Thrombozytenkonzentrat beschrieben worden, welches zur Behandlung von Sehnen- und Bänderverletzungen in der Pferd -und Humanmedizin verwendet wurde. Jüngste Studien deuten an, dass diese Therapieform die Sehnenheilung effektiver als andere Thrombozytenkonzentrate fördern könnte. Allerdings ist der Effekt dieses Therapieansatzes auf die Sehnenheilung unbekannt. Daher war das Ziel dieser Studie, die Auswirkungen der intraläsionalen Behandlung von Sehnenverletzungen mit ACP zu bewerten. Bei acht gesunden Pferden wurden an der oberflächlichen Beugesehnen (OBS), an beiden Vorder- und Hintergliedmaßen, standarisierte chirurgische Läsionen gesetzt. An den Tagen 7 und 15 nach Induktion der Läsion wurde an je einer Vorder- und Hintergliedmaße unter Ultraschalkontrolle ACP intralesional injiziert. Die jeweilige kontralaterale Gliedmaße wurde mit steriler Kochsalzlösung behandelt. Für diese Studie wurden nur die Daten der Vordergliedmaßen verwendet. Der Verlauf der Sehnenheilung wurde klinisch und mit Graustufen- bzw. Farbdopplersonographie kontrolliert. Nach 24 Wochen wurden die Sehnenproben für die Bewertung der biochemischen, biomechanischen und histologischen Parametern entnommen. Dreiundzwanzig Wochen nach der Behandlung wiesen die ACP behandelten Sehnen eine deutlich geringere Konzentration an Glykosaminoglykanen (GAG) ($p \leq 0.05$) im Vergleich zur Placebogruppe. Während der Proliferationsphase der Heilung führen intraläsionale ACP-Behandlungen von chirurgisch induzierten „Core Lesions" der OBS, im Vergleich zur Placebogruppe, zu einem begrenzten Effekt in der Sehnenheilung. Langfristige Placebo-kontrollierte klinische Studien mit einer höheren Anzahl von Pferden sollten durchgeführt werden, um nachzuweisen, ob dieser Effekt klinisch relevant ist.

Schlüsselwörter: Pferd / Trombozytenkonzentrat / Autologous Conditioned Plasma (ACP) / Sehnen / Heilung / Model / Orthopädie

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Introduction

Tendon pathologies are among the most common musculoskeletal disorders in horses (*Avella* et al. 2009, *Singer* et al. 2008, *Murray* et al. 2006, *Kasashima* et al. 2004). After injury the tendon repairs by forming disorganized scar tissue (*Birch* et al. 1998, *Williams* et al. 1980), which is of inferior functional quality when compared to normal tendon and therefore prone to re-injury (*Crevier-Denoix* et al. 1997). Even though some therapeutical approaches have shown promising results (*Godwin* et al. 2012), many of the currently available treatment modalities cannot significantly reduce these high recurrence rates (O'Meara et al. 2010, *Dyson* 2004, *Gibson* et al. 1997).

During the last years the use of autologous blood products for treatment of equine tendon and ligament injuries has increased (*Bosch* et al. 2010, *Waselau* et al. 2008). Platelet concentrates seem to be a promising option, as upon thrombocyte granules activation a large number of anabolic growth factors (TGF-ß, PDGF-BB, VEGF, etc.) are released. These growth factors are supposed to act as humoral mediators and biological catalysts, promoting tissue healing (*Boswell* et al. 2012). Moreover, recent studies indicate that platelet concentrates exhibit an important in vitro and in vivo anti-inflammatory effect when used to treat tenocytes exposed to an inflammatory insult and tendon injuries (*Zhang* et al. 2013).

At the moment there is no consensus as to which platelet concentrate preparation is most efficient for the improvement of tendon healing. There are a variety of preparations featuring different profiles concerning growth factor concentration, kinetics of growth factor release, inflammatory cytokine expression, platelet and white blood cell counts (*McCarrel* et al. 2012, *Sundman* et al. 2011). These variations are likely to have an impact on the clinical outcome and hence the effects of specific platelet preparations on tendon healing need to be investigated. Autologous Conditioned Plasma (ACP, Arthrex Inc.) is a leukocyte-reduced platelet concentrate that has been used in equines and humans for the treatment of tendon and ligament injuries (*Rindermann* et al. 2010, *Deans* et al. 2012). In vitro studies have shown that ACP allows concentration of platelets and growth factors while at the same time decreasing WBC counts (*McCarrel* et al. 2012, *Kissich* et al. 2012). Even though there are conflicting reports about the role that WBC counts in blood products play on tendon healing (*McCarrel* et al. 2012, *Sundman* et al. 2011, *Everts* et al. 2006), several in vitro studies have demonstrated that lower WBC counts in the platelet concentrates are directly correlated to a lower concentration of catabolic enzymes (*McCarrel* et al. 2012, *Sundman* et al. 2011). Therefore, it has been suggested that leukocyte-reduced platelet concentrates might be superior than platelet rich plasma (PRP) in stimulating tendon healing, since persistent inflammation incited by the high concentration of WBC may result in scar tissue formation (*McCarrel* et al. 2012).

The aim of this study was to evaluate the effects of the ACP on healing of standardized surgically induced SDFT core lesions in horses. It was hypothesized that intra-lesional ACP treatments at days 7 and 15 after SDFT lesion induction would improve sonographic, biochemical, biomechanical and histological parameters of the tendons when compared to saline.

Materials and Methods

SDFT lesions affect more often the forelimbs than the hindlimbs, therefore, for this paper, only the results of the forelimbs will be discussed. The data derived from the hindlimbs was used to compare the healing differences between saline treated fore- and hindlimb SDFT core lesions in tendinopathy models (*Estrada* et al. 2014).

Experimental animals

Eight, mixed breed, 2.5 to 6 year old horses with average weight of 434 (± 38) kg were selected. The animals were free of lameness and presented no clinical or ultrasonographic signs of acute or chronic SDFT lesions. Horses were housed in individual boxes, fed a maintenance ration of concentrate with hay and had water ad libitum. This study was approved by the Animal Welfare Committee (Session Nr. 003-10), School of Veterinary Medicine, National University, Costa Rica in accordance to the Costarrican Act 7451 on Animal Welfare.

Lesion induction and postoperative management

Tendon lesions were created under general anesthesia in the SDFT of all fore- and hindlimbs using a modification of the tendinitis model described by *Schramme* and others (2010). The horses were sedated with xylazine (Procin Equus: Pisa, Mexico) (1,1 mg/kg bwt, i.v.) and then induced using ketamine/midazolam (Ketamid: Holliday, Argentina) (2,2 mg/kg bwt, i.v.). Thereafter, the anesthesia was maintained with isoflurane (Aerrane: Baxter Int., Illinois). A linear array probe (DP 3300 Vet: Mindray, China) covered with a sterile palpation sleeve was used to standardize the incision site just proximal to the digital flexor tendon sheath, in the ecographic zones 3A and 4A, in the fore- and hindlimb, respectively (*Rantanen* et al. 2011). A 1.5cm skin incision was performed in the selected site using a scalpel blade No.10 and then a stab incision was made through the paratendon with a scalpel blade No.15. A new disposable 3.5 mm motorized synovial resector (Razorcut: Smith and Nephew, USA) was used for each horse. Using ultrasonographic guidance, the inactivated synovial resector was introduced into the core of the tendon in a proximal direction over a length of 7 cm. Once in position, the synovial resector was activated and slowly retrieved in approximately 20 s. The paratendon and skin were closed using standard technique. The horses were managed with phenylbutazone (Lisan, Costa Rica) (2.2 mg/kg bwt p.o. twice daily) and penicillin-streptomycin (Pen-Strep: Norbrook, Ireland) (1 ml/25 kg bwt i.m. once daily) 1 hour pre-operatively and 3 days post-operatively. During the first 3 weeks, the animals were box-rested and the distal limbs were immobilized using a regularly changed Robert Jones bandage. From week 4 onwards, a controlled exercise program, based on a previously described protocol (*Bosch* et al., 2010) was initiated (Table 1).

ACP treatment and sampling protocol

Intralesional treatment was performed 1 and 2 weeks post-surgery. Ten milliliters of whole blood without anticoagulant were aseptically withdrawn from the left jugular vein directly

to the ACP kits. The samples were centrifuged at 189 g for five minutes. The animals were sedated using xylazine (Procin Equus: Pisa, Mexico) (1.1 mg/kg bwt i.v.). The injection site was aseptically prepared and desensitized using 1 ml of subcutaneous lidocaine (Faryvet, Costa Rica). Using ultrasonographic guidance, 2.5 ml of either ACP or sterile saline solution were injected into the core of lesions by a clinician blinded to treatment. The tendons were injected, once per treatment session, using a 20G × 1½ inch needle. The procedure was performed on weight-bearing and the needle was introduced in a longitudinal plane, in a plantaroproximal-dorsodistal direction, aiming at the proximal third of the lesions. Assignment of the ACP-treated limb in each fore and hindlimb pair was random. The procedure was repeated on week 2, therefore freshly prepared ACP was also used for the second intra-tendinous injection.

Cell count and growth factor quantification

The remaining ACP was divided in four to five 0.5 ml EDTA vials. One fresh 0.5 ml ACP aliquot together with a whole blood sample from each animal were examined using an hematology analyzer (Poch100-i: Sysmex, Japan). The rest of the ACP aliquots from the treatment sessions on weeks 1 and 2 and one plasma sample of each animal from the session on week 1 were frozen at -20°C until the active concentrations of PDGF-BB and TGF-B were quantified using sandwich ELISA (Quantikine: R&D Systems, USA).

Clinical assessment

The vital parameters, weight bearing, presence of lameness at walk, local swelling and sensitivity were monitored daily throughout the study. Twenty-four weeks following lesion induction, the lameness was graded using the AAEP lameness score (24) and tendon tenderness was classified as normal, mild, moderate and severe.

Gray-scale ultrasonographic evaluation

A linear multi-frequency probe set at 10 MHz (DP 3300 Vet: Mindray, China) and a silicone standoff pad were used to perform the ultrasonographic examinations of the SDFT lesions at 1, 2, 4, 6, 10, 15 and 24 weeks post-surgery. The limbs were divided in transverse and longitudinal zones as described in the literature (*Rantanen* et al. 2011) and the distance distal to the accessory carpal bone or calcaneal tuberosity was recorded for each zone. Re-examinations of every zone in each pair of fore- and hindlimbs were performed at the same distances. A semi-quantitative scoring system was used to grade the echogenicity and fiber alignment of the lesions (*Rantanen* et al. 2011). The lesion length (LL) was defined as the length from the most proximal to the most distal aspect of the lesion. The total tendon cross-sectional area (TT-CSA) was calculated summing the CSA of the SDFT in 6 different zones (1A to 3B or 2A to 4B). The total lesion cross-sectional area (TL-CSA), total echo-score (T-ES) and total fiber alignment score (FAS) of transverse zones presenting a lesion were calculated for each tendon summing the values of each parameters to get total values. The total lesion percentage (TL-%) was calculated as follows: (TL-CSA/TT-CSA)*100 (*Rantanen* et al. 2011). A clinician blinded to treatment performed the data acquisition, measurements and scoring of the images (RJE).

Table 1 Exercise protocol of the horses after the lesion induction of the superficial digital flexor tendons

Week Post-Surgery	Walk (min/day)	Trot (min/day)
1-3	-	-
4-6	10	-
7-10	20	-
11-14	30	-
15-20	40	-
21-22	35	5
23-24	30	10

Intra-tendinous vascularization assessment

The intra-tendinous vascularization was scored using the technique reported by *Bosch* and others (2011a). Color Doppler Ultrasound (CDU) scans were performed 24 weeks post-surgery. The limbs were scanned in flexed position to relax the tendinous structures and therefore avoid the collapse of intratendinous vessels due to mechanical forces. The images were obtained using a multi-frequency linear array probe set at 10 MHz and machine settings suitable for low flow vessel detection (VEL/6.2 MHz; 0 Db; 1,099 KHz PRF) (Acuson Antares: Siemens, Germany). The lesions were localized and scanned from lateral to medial on the longitudinal plane. The image sequences of the each scan were stored and the frame with the highest vascularization was subjectively selected and then scored. Data acquisition and vascularization grading was performed by a clinician blinded to treatment (RJE).

Sample harvesting, handling and shipping

After 24 weeks the horses were euthanized. The animals were induced using a combination of xylazine (Procin Equus: Pisa, Mexico) (1.1 mg/kg bwt i.v.) and ketamine-midazolam (Ketamid: Holliday, Argentina) (2 mg/kg bwt, i.v.); once a deep anesthetic plane was achieved a bolus of an oversaturated magnesium sulphate solution (1 g/kg bwt i.v.) was administered. The SDFTs were harvested immediately after the euthanasia and divided in different sections as reported by Bosch and others (*Bosch* et al. 2010). In all the horses the lesion were inside the paratendon and easily distinguishable from the healthy tendon (Fig 1). In brief, a transverse 1 cm tendon slice was obtained 2 cm proximal to the scar of the stab incision in the paratendon. The core lesions were identified and

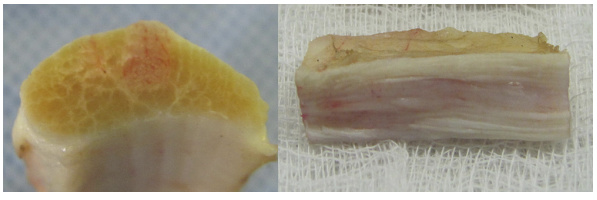

Fig 1. Transverse and longitudinal images of a surgically induced core lesions in the SDFT 24 weeks post-surgery.

a 4 mm punch, directed longitudinally, was used to harvest injured tissue only. These samples were divided in 3 sections that were lyophilized for 24 h using a vacuum freeze-dryer and then used to perform the biochemical analysis. Proximal to the aforementioned tendon slice, a 3 cm segment was harvested and divided through the center of the lesion in two longitudinal sections. The half used for biomechanical tests was initially frozen at -80 °C, then shipped to the laboratories using a dry shipper (MVE Vapor Shipper: Chart Industries, USA) at approximately -150 °C and subsequently stored at -80 °C until further processing. The other half used for histological evaluation was fixed in 4 % formalin for 48 h, placed in 96 % ethanol for 5 days (*Bosch* et al. 2010). These samples were then sequentially transferred through a series of solutions of increasing isopropyl alcohol concentration and then embedded in paraffin blocks.

Glycosaminoglycans and DNA quantification

The lyophilized tendon segments were papain digested (0.1 IU/mg of dwt) at 56 °C for 12 hours. A dilution of the papaine digest of each tendon was used to perform the GAG and DNA assays. The quantification of the sulphated GAGs was performed using the 1,9-dimethylmethylene blue dye assay (*Farndale* et al. 1982). Shark chondroitin sulphate was used as a standard (0–100 g/ml). After an incubation period, the plates were assessed on a microplate reader (VersaMax: Molecular Devices, USA). Total DNA was quantified by means of the reaction with fluorescent dye Hoechst 33258 (*Kim* et al. 1988). Salmon sperm DNA was used as a standard (0–20 g/ml). The luminescence was measured using a fluorescence spectrometer (LS-50B: Perkin Elmer, USA). In both cases the final results were expressed as g/mg of dry weight tendon.

Degraded collagen, total collagen, and cross links quantification

The tendon samples were processed with -chymotrypsin to digest the denatured collagen (*Lin* et al. 2005). Hydroxyproline (Hyp) concentrations in the supernatant (containing the degraded collagen) were determined after the reaction with chloramine T and dimethylaminobenzaldehyde (*Reddy* and *Enwemeka* 1996). Results were calculated as previously described and expressed as percentage of degraded collagen (*Lin* et al. 2005). After the -chymotrypsin digest, the tendon explants (containing the intact collagen) were hydrolyzed at 110 °C for 24 h. The samples were vacuum dried for 24 h, diluted in ultrapure water and centrifuged at 13,000 g for 20 min. The obtained supernatant was submitted to mass spectrometry (MS), to determine the concentrations of hydroxyproline (Hyp), hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) using a technique reported by *Bosch* and others (2010). For calculation of the total collagen the Hyp content in the -chymotrypsin digest supernatant was summed to the Hyp of the explants measured with the MS.

Pyrrole quantification

Pyrrole was quantified using an adaptation of a previously reported method (*Thorpe* et al. 2010). The freeze-dried tissues were minced, suspended in a pepsin/HCl solution and digested in a water bath at 45 °C for 36 h. Thereafter the samples were centrifuged at 12,000 g for 15 min and 200 l of supernatant were mixed with 40 l of Ehrlich s Reagent (4-dimethyaminobenzaldehyde, perchloric acid and deionized water) in a microplate. The samples were incubated at room temperature for 10 min and the absorbance was measured at 558 nm and 650 nm (non-related wavelength) in a microplate reader (VersaMax, Molecular Devices, USA). The pyrrole concentrations were calculated by comparison with a reference line prepared mixing 200 l of 1-methyl-pyrrole (0-20 mol/l) with 40 l of Ehrlich s Reagent. Results were expressed as mol per mol of collagen.

Biomechanical assessment

The biomechanical properties of the tendon samples were assessed using a modification of the method reported by *Bosch* and others (2010). The samples were thawed at room temperature. Longitudinal segments with an approximate cross section of 4 mm^2 and a length of 3 cm were cut from the core lesion of each tendon sample with a cutting device consisting of four disposable high profile microtome blades (Feather: Safety Razor, Japan) at distances of 2 mm. A material testing machine (AX M250–2.5 kN: Testometric Company, UK) was used for failure testing. Sand paper was placed between the ends (proximal and distal) of the tendon sample and the clamps of the machine to decrease slippage. Once in position, the depth and width of the midsection of each sample were measured with a 0.01 mm resolution electronic caliper in a transverse plane to calculate cross-sectional area. The selected segments were tensed and preconditioned at 1 Hz and 3 % strain for seven cycles and then tested to failure at a speed of 6 mm/min. The force at failure (Fmax) and the stress-strain curve were determined for every sample. In each case the ultimate tensile strength (UTS) was calculated (Fmax/tendon cross-sectional area) and the elastic modulus (EM) deduced from the slope of the linear part of the curve (*Bosch* et al. 2010).

Histology

Longitudinal 5 m thick tendon sections were stained with Masson's Trichrome (Artisan: Dako, Denmark). When using this commercial histochemistry staining, the organized tendon collagen was stained red and reparative tissue blue, as previously reported by *Cadby* and others (2013). Microphotographs of five consecutive fields of view (1x magnification) from different locations of each tendon samples were stored. At this magnification the complete thickness of the tendon section was observable in each microphotograph. An image manipulation software (Gimp 2.8: GNU Project, USA) was used to increase the contrast of the pictures. The red/blue ratio of each histological section was calculated using an image-processing package (Fiji / ImageJ: National Institute of Health, USA). Briefly, the images were assessed setting a threshold that allowed the isolation of the different stained areas in the histological section. Thereafter, the areas of interest (red/blue) were measured (*Jensen* 2013). The ratio between them calculated was calculated per image and then the average red/blue ratio of the five images of each sample was calculated. The aforementioned color ratio reflects the ratio

between organized and reparative tissue and therefore the degree of damage presented in the tendon.

Statistical analysis

The data was analyzed using Excel (Microsoft Corporation, USA) and Graph Pad Prism 6 (GraphPad Software, USA). The D'Agostino-Pearson test was used to determine the data distribution. A paired Student's t-test was used to analyze the CDU vascularization score and the biochemical, biomechanical and histological parameters. After passing normality tests, the sonographic measurements were evaluated with repeated measures two-way ANOVA followed by a Bonferroni s multiple comparison test. The sonographic scores showed a nonparametric distribution and therefore a Friedman test followed by Dunn s multiple comparison test was applied. The significance level was set at $p \leq 0.05$. Results were reported as mean ± SD.

Results

Cell count and growth factor quantification

There was no difference in platelet counts between the ACP and whole blood ($P = 0.85$), but there was a significant decrease of WBC counts in ACP (28.8 times). The TGF- concentrations in ACP were not significantly increased when compared to blood plasma ($P = 0.49$), but levels of PDGF-BB were significantly higher (6.1 times) (Table 2).

Clinical assessment

After lesion induction the horses bear weight normally, were not lame at walk and the SDFT presented a moderate to severe tenderness at palpation. No post-surgical complications were noted. After 24 weeks, two horses presented a grade 2/5 lameness (one forelimb and one hindlimb) and were therefore hand-walked for 10 min per day until the end of the study. The horse with the forelimb lameness presented mild tendon tenderness in the lame forelimb (ACP treated) at week 24. In the case of the hindlimb (saline treated), the SDFT showed no tenderness and no further pathological changes that could explained the lameness were found.

Ultrasonographic assessment

All horses developed core lesions that presented ultrasonographic appearance of SDFT naturally occurring injuries (Fig 2). The grey-scale ultrasonographic parameters evaluated throughout the study didn t show any significant differences (Fig 3–4). Twenty-three weeks after treatment, the ACP treated tendons presented a higher color Doppler vascularization score (2.62 ±0.91) when compared to saline treated

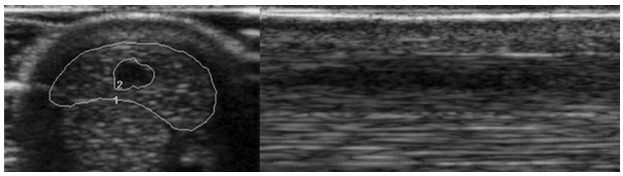

Fig 2 Transverse and longitudinal ultrasonographic images of a surgically induced core lesion in the SDFT 15 days post-surgery.

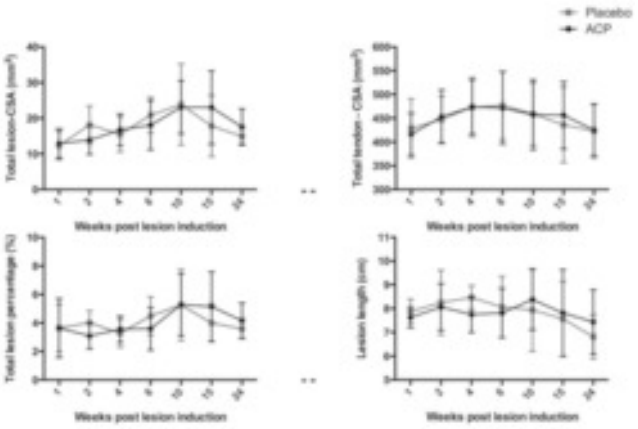

Fig 3. Gray-scale ultrasonography measurements (mean ± SD) of autologous conditioned plasma (ACP) and saline-treated surgically induced core lesions in the superficial digital flexor tendon (SDFT) over a 24-week period. (a) Total lesion cross-sectional area (TL-CSA) (b) total tendon cross-sectional area (TT-CSA) (c) total lesion percentage (TL-%) and (d) lesion length (LL).

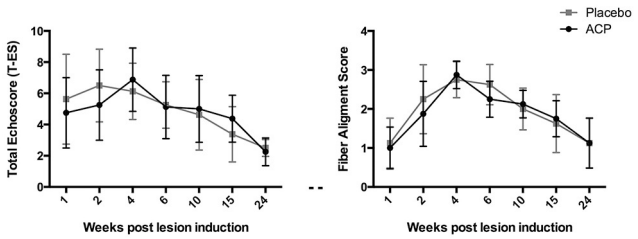

Fig 4 Gray-scale ultrasonography semi-quantitative scores (mean ± SD) of autologous conditioned plasma (ACP) and saline-treated fore- and hindlimb surgically induced core lesions in the superficial digital flexor tendon (SDFT) over a 24-week period. (a) Total echoscore (T-ES) and (b) fiber alignment score (FAS).

Table 2 Hematological parameters in whole blood and ACP and growth factor concentration in conventional plasma and ACP

	Average		p-Value
Parameter	Whole Blood (n=16)	ACP (n=16)	
Platelets (x10^3 /µl)	165.3 ± 51.6	162.0 ± 43.6	0.79
Leukocytes (x10^3 /µl)	9.8 ± 2.6	0.34 ± 0.18	< 0.0001*
	Plasma (n=8)	ACP (n=16)	
TGF-β - ng/ml	8.134 ± 1.051	8.752 ± 2.330	0.49
PDGF-BB - ng/ml	0.066 ± 0.069	0.405 ± 0.194	0.0001*

TGF, transforming growth factor; PDGF, platelet derived growth factor. * $p \leq 0.05$

tendons (2.00 ± 1.30). Nevertheless, this difference showed only a significant trend (P = 0.09).

Biochemical assessment
The ACP treated tendons presented a significantly lower GAG content, when compared to placebo (P ≤ 0.05). Other compositional parameters did not differ significantly (Table 3).

Biomechanical assessment
Neither the ultimate tensile strength (P = 0.93) nor the elastic modulus (P = 0.13) differed significantly, when comparing ACP and placebo. One set of data was lost since it was accidentally not stored in the system drive (Table 3).

Histology

The red (organized tissue)/blue (reparative tissue) ratio of microphotographs of five consecutive fields of view of the tendon histological sections stained with Masson s Trichrome showed no significant difference between treatments (P = 0.95) (Table 3).

Discussion

Our results indicate that ACP treatment in surgically induced core lesions has a limited influence on tendon healing when compared with saline. In this study, the majority of the ultrasonographic, biochemical, biomechanical and histological parameters showed no significant differences between treatments. Nevertheless, the significant decrease of sulphated GAGs in the ACP treated tendons can be interpreted as a limited, but possibly beneficial, effect on tendon healing.

There are conflicting opinions about the role of leukocytes in blood products used to treat tendinopathy and how they affect tendon healing (*McCarrel* et al. 2012, *Sundman* et al. 2011, *Everts* et al. 2006). There are indications, that in vivo, the leucocytes may have positive effects, such as promote healing, modulate the immune response, anti-inflammatory action and release of anti-microbial substances (*Everts* et al. 2006). On the other hand, recent studies have claimed that platelet concentrates with reduced leukocyte concentration might be a suitable option for improving tissue repair, since high leukocyte counts in PRP induce an increase in the production of catabolic and inflammatory cytokines (*McCarrel* et al. 2012, *Sundman* et al. 2011). It has been hypothesized that this feature might play a negative role on tendon healing, stimulating more scar tissue formation (*McCarrel* et al. 2012). The ACP without anticoagulant used in this study showed a significant reduction of the WBC counts, but no measurable increase of the platelet counts. Nonetheless, Wright stains performed on several smears of ACP samples showed extensive platelet aggregation. Due to this finding, we believe that the lack of initial anticoagulant in ACP kits caused a sequestration of the platelets in the aggregates, explaining the relatively low platelet counts in the ACP as the hematology analyzer will have been unable to count the platelets accurately under these circumstances. This hypothesis is also supported by the fact that the PDGF-BB values were significantly increased in the ACP samples, which can only be explained by concentration of the platelets. On the other hand, recent studies suggest that the ACP kit might not be as efficient concentrating platelets as it was previously reported (*Hessel* et al. 2014). This might be an easier explanation for the low platelet counts, but would not explained the increase in PDGF-BB concentration. However, our study can not be directly compared to Hessel's since ACD-A was not used in the ACP kits. Interestingly, other studies have used ACD-A in the ACP kits (*Kissich* et al. 2012), but in spite of the usage of anticoagulants an important variability in the average platelet concentration was seen, when compared to the data published by *Hessel* and others (2014).

This study used ACP without anticoagulant, as it has been shown that growth factor concentrations are higher in serum than in anticoagulated blood (*Zimmermann* et al. 2005). Interestingly, the PDGF- BB concentrations achieved in this study were lower and the TGF- were higher (but not significantly higher than conventional plasma) than the reported values using anticoagulant citrate dextrose solution A (ACD-A) (*Hessel* et al. 2014, *Kissich* et al. 2012). Several studies have

Table 3 Biochemical, biomechanical and histological parameters of ACP and Placebo treated SDFTs 23 weeks after treatment.

Parameters	Treatment Group		p-Value
Biochemical	Placebo (n=8)	ACP (n=8)	
GAG - µg/mg dwt	37.21 ± 24.78	26.98 ± 14.86	0.05*
DNA - µg/mg dwt	3.42 ± 0.69	3.32 ±1.32	0.81
Total Collagen - mg/mg dwt	0.63 ± 0.01	0.70 ± 0.10	0.34
Degraded Collagen - %	0.89 ± 0.61	0.93 ± 0.38	0.91
Pyrrole - mol/mol collagen	0.053 ± 0.01	0.043 ± 0.01	0.11
HP - mol/mol collagen	0.52 ± 0.09	0.59 ± 0.16	0.19
LP - mol/mol collagen	0.022 ± 0.005	0.020 ± 0.006	0.51
Biomechanical	Placebo (n=7)	ACP (n=7)	
Ultimate Tensile Stress – MPa	15.20 ± 9.44	14.89 ± 4.57	0.94
Elastic Modulus- GPa	0.147 ± 0.07	0.103 ± 0.03	0.13
Histology	Placebo (n=8)	ACP (n=8)	
Masson´s Trichrome Red / Blue Ratio – Decimals	0.164 ± 0.087	0.161 ± 0.092	0.95

GAG, glycosaminoglycans; HP, hydroxylysylpyridinoline; LP, lysylpyridinoline * p ≤ 0.05

demonstrated that EDTA may alter the determination of the growth factor concentration (*Zimmermann* et al. 2005, *Bielohuby* et al. 2013). In this study, the storage of the ACP samples in EDTA vials may have affected the accuracy of the determination of the growth factor concentrations. Nevertheless, as conventional plasma and ACP samples were treated identically, there is no doubt about the highly significant increase in PDGF-BB concentration in ACP samples. Even though there was a significant difference in the PDGF-BB concentration, these results were significantly lower when compared to previously reported values (*Hessel* et al. 2014, *Kissich* et al. 2012). Interestingly, a recent study suggests that ACP is apparently not as efficient concentrating PDGF-BB and TGF- as it was previously reported (*Hessel* et al. 2014), this might suggest that the growth factor differences found in these 3 studies could have been caused by a low repeatability of the ACP kit when using horse blood. This is especially true when comparing the values reported by *Hessel* and others (2014) and *Kissich* and other (2012), where ACD-A and a very similar processing protocol were used.

Glycosaminoglycans (GAGs) are polysaccharides that are usually covalently attached to core protein, forming macromolecules known as proteoglycans (*Esko* 1999). In tendons, they are in part involved in the regulation of the collagen fibrillogenesis, affecting fibril size and formation rate (*Cribb* and *Scott* 1995). Moreover, these molecules contribute to the biomechanical and structural properties of the extracellular matrix (*Parkinson* et al. 2011). The role of GAG accumulation after tendon damage is seen controversial in the literature. Previous studies in humans and equines indicate that degenerated tendon regions present higher concentrations of sulphated GAGs (*Birch* et al. 1998, *Parkinson* et al. 2010). Synthesis and accumulation of these molecules is usually correlated to an increased cellular metabolism within injured areas in the tendon (*Parkinson* et al. 2010) and a marked increase of small collagen fibers, decreasing the mechanical properties of tendons and ligaments (*Halper* et al. 2006). On the other hand, a recent study using blood products for the treatment surgically induced core lesions interpreted the increase of intralesional sulphated GAG concentration as a positive feature of tendon healing (*Bosch* et al. 2010). The GAG values found in this study were significantly higher than the average GAG content reported for normal equine SDFTs in the metacarpal region (*Lin* et al. 2005), but similar to what has been reported for SDFT core lesions 24 weeks after induction (*Bosch* et al. 2010). The fact that the ACP treated tendons showed significantly less sulphated GAGs suggests that the treatment might have positively affected the intra-tendinous cellular metabolism, which may thus have a beneficial effect on the characteristics of both the non-collagenous and collagenous matrix of the tendons. The difference in the GAG

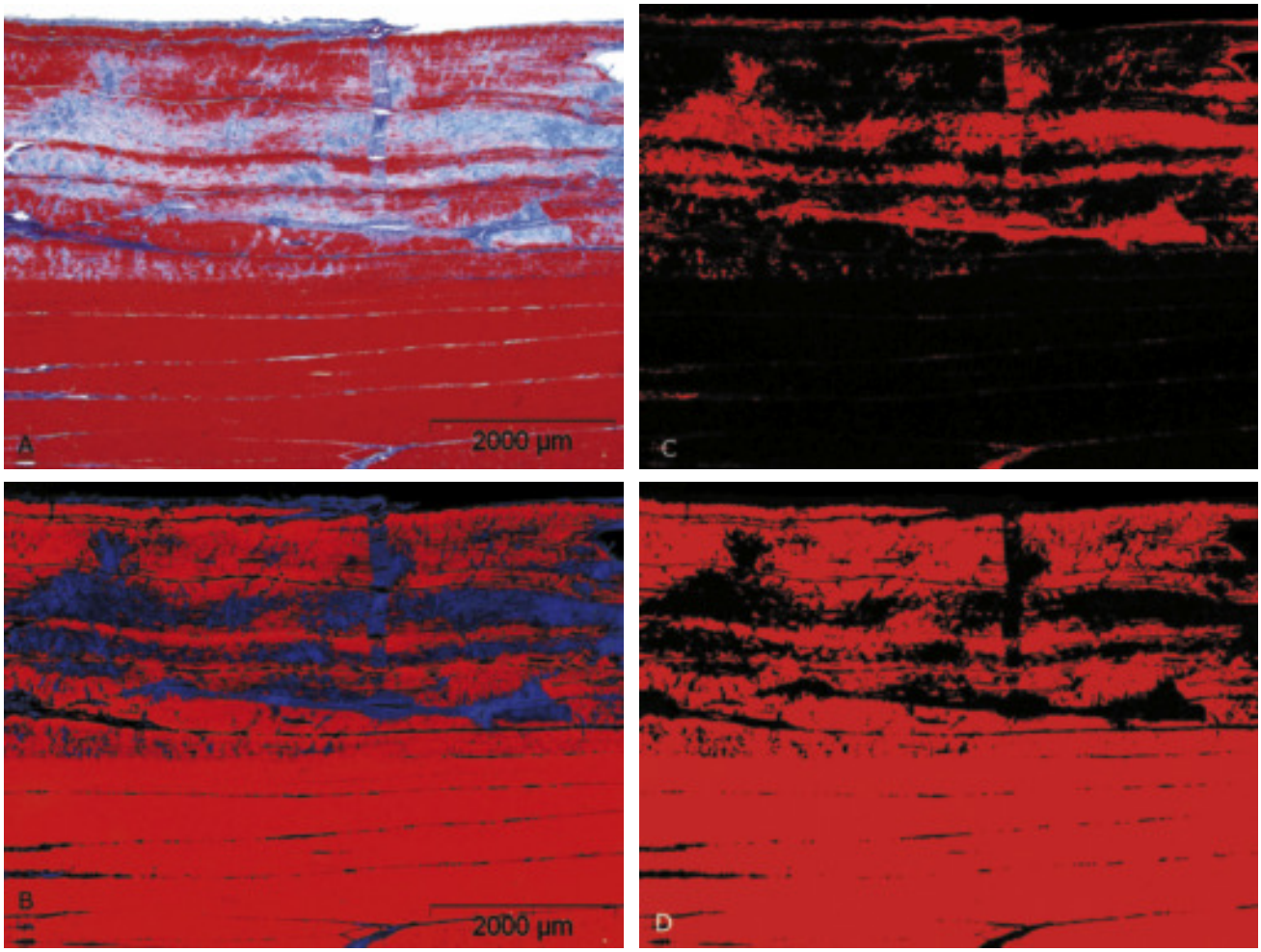

Fig 5 (A) Example of an histological section of a placebo treated tendon stained with Masson s Trichrome. The red areas mainly represent organized collagen and the blue reparative tissues (B) Manipulation of the image to increase contrast using an image manipulation software (Gimp)q . (C-D) Determination of the percentage of the red and blue stained regions for the calculation of a red/blue ratio using an imaging processing package (Fiji/ImageJ)r.

content may also be interpreted as a faster tendency towards normalization of this parameter when compared to control treated SDFTs. Nonetheless, this interpretation might be daring, without a direct comparison of the GAG content between injured and healthy tendon sections and without having any other significant difference in the rest of the biochemical parameters. The biomechanical tests performed in this study could not demonstrate a significant difference between the mechanical properties of the ACP and saline treated tendons. Therefore, it is most likely that the significant difference in GAG content found in this study does not play a major role affecting the biomechanical characteristics of the tendons.

Tendon lesions present an increased blood flow after acute injury, allowing cell recruitment and providing humoral mediators, growth factors and nutrients needed for adequate lesion healing (*Yang* et al. 2012). There is a general consensus that normal equine tendons present no detectable intratendinous blood flow when evaluated with color or power Doppler ultrasound (*Bosch* et al. 2011, *Kristoffersen* et al. 2005). The role of persistent intra-tendinous vascularization is seen controversial in the literature. Traditionally, persistent vascularization has been interpreted as a sign of inadequate healing and incomplete repair (*Kristoffersen* et al. 2005, *Murata* et al. 2012), but it is not clear in the literature for how many months the vascularization should be visible, so that it can be called persistent. On the contrary, *Bosch* and others (2011a) reported that 23 weeks after intra-tendinous PRP treatment, the PRP treated tendons presented a higher CDU vascularization score than saline treated tendons. This finding was interpreted as a positive feature of the therapy and it was suggested that this might be in part responsible for the improved quality of the repaired tendon tissues obtained in this study. Twenty-three weeks after treatment, the ACP treated tendons presented a borderline significant trend towards a higher vascularization score when compared to the saline treated structures. The increased CDU vascularization score is probably correlated to an increased neovascularization, most likely stimulated by the growth factors injected in the tendon lesion. This result might be interpreted as positive feature of this therapeutical approach, since adequate vascularization is a prerequisite for optimal progress of the acute and proliferative phases of healing (*Bosch* et al. 2011a). However, future studies must investigate the risks of inducing persistent vascularization when using intra-tendinous platelet concentrates and the role that the persistent vascularization plays in chronic equine tendinopathies.

The Masson s Thrichrome stain used in this study stained the organized tendon fibers red, whereas the reparative tissue blue, as previously reported (*Cadby* et al. 2013). This pattern was consistent in all samples, allowing making a clear distinction between blue and red areas. This staining technique, in conjunction with the imaging analysis system, proved very helpful for an objective global assessment of the tendon structure (Fig 5).

It has been suggested that leukocyte-reduced platelet concentrates might be superior than PRP stimulating tendon healing, since persistent inflammation incited by the WBC may result in scar tissue formation (*McCarrel* et al. 2012). Comparing our results to those of other studies using similar models (*Bosch* et al. 2010, *Bosch* et al. 2011a, *Bosch* et al. 2011b), it seems that PRP has a more profound effect on tendon healing than ACP without anticoagulant. On the other hand, even though PRP showed several positive effects on tendon healing, it is still questionable if the increased intratendinous vascularization, sulphated GAG content and cellularity found 23 weeks after the first treatment can be interpreted as beneficial, since these features have been previously correlated to poor tendon repair (*Godwin* et al. 2012, *Kristoffersen* et al. 2005, Birch et al. 1998). Therefore, it is of paramount importance to continue studying the in vivo effect of the different platelet preparations to determine which is more efficient normalizing biomechanical, biochemical and histological parameters towards those levels found in healthy tendons.

There are limitations to this study. Even though this surgical tendinopathy model created lesions emulating naturally-occurring SDFT core lesions (*Cadby* et al. 2013), the fiber disruption caused by the synovial resector does not replicates the degenerative process that is believed to precede the majority of the natural clinical injuries (*Birch* et al. 1998). A period of 24 weeks is certainly not long enough to evaluate the end stage tendon healing, since the final maturation of tendon repair takes approximately one year (*Silver* et al. 1983). Histological evaluations of tendon healing have been traditionally performed using semi-quantitative scores (*Bosch* et al. 2010). Aiming at objectivizing this evaluation, an image-processing package was used to analyze the microphotographs of the histological sections in this study. The fact that only this technique was used might have cause a loss of valuable subjective information. Even though the normal biochemical parameters of the SDFT have been already published (*Lin* et al. 2005), processing healthy regions of the tendons would have allowed a more accurate comparison between the lesion site and the normal tissues. The use of a multi-limb model might have affected the loads on the injured tendons and hence the healing process. However the horses in this study never presented overt signs of pain or showed an abnormal weight bearing, therefore is not likely that this factor played a role in the healing of the lesions.

Conclusions

Our study indicates that 2 intra-tendinous ACP treatments (without anticoagulant), during the proliferative phase of healing, in surgically induced SDFT core lesions, have a limited effect on tendon healing when comparing ultrasonographic, biochemical, biomechanical and histological parameters with the control treatment. Long-term placebo controlled clinical trials with more horses are warranted to determine if this effect is clinically significant.

Conflict of interests

Arthrex Inc. financed approximately 30% of the costs of this study, but it was not involved in the collection, analysis or interpretation of the data or in the publication of the manuscript. The authors have no competing interests that could influence the content of this paper.

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9.1.2. Comparison of healing of fore- and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon.

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Comparison of healing in forelimb and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon

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Keywords
Horse, superficial digital flexor tendon, tendinopathy, model, healing

Summary

Objective: Even though equine multi-limb tendinopathy models have been reported, it is unknown if fore- and hindlimb tendon healing behave similarly. The aim of this study was to compare the healing process of surgically induced superficial digital flexor tendon (SDFT) core lesions of fore- and hindlimbs in horses.

Methods: Tendon core lesions were surgically induced in the SDFT of both fore- and hindlimbs in eight horses. One randomly assigned forelimb and one randomly assigned hindlimb were injected with saline one and two weeks post-surgery. The healing process was monitored clinically and ultrasonographically. After 24 weeks, the tendons were harvested and biochemical, biomechanical and histological parameters were evaluated.

Results: Twenty-four weeks post-surgery, the forelimb SDFT lesions had a significantly higher colour Doppler ultrasound vascularization score ($p = 0.02$) and glycosaminoglycan concentration ($p = 0.04$) and a significantly lower hydroxylysylpyridinoline content ($p = 0.03$).

Clinical relevance: Our results indicate that fore- and hindlimb SDFT surgically induced lesions exhibit significant differences in several important parameters of tendon healing 24 weeks post-surgery. These differences create significant challenges in using all four limbs and accurately interpreting the results that one might generate. Therefore these findings do not support the use of four-limb models for study of tendon injury until the reasons for these differences are much better understood.

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Introduction

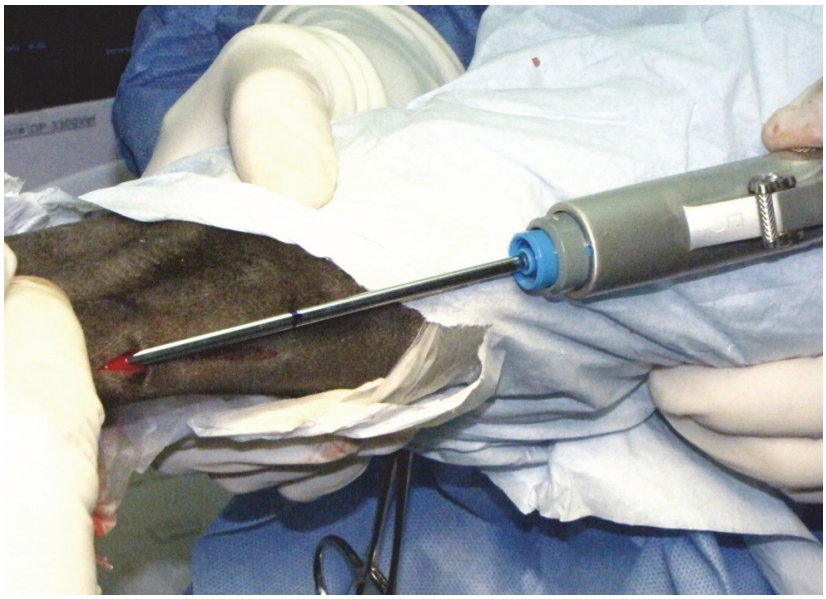
Tendon pathologies are among the most common musculoskeletal disorders in horses (1). Some tendons are more prone to injury than others and the superficial digital flexor tendon (SDFT) is one of the most commonly affected structures (1–4). Tendon lesion distribution varies between horses engaged in the different equestrian disciplines, but in general SDFT lesions affect the forelimbs more often than the hindlimbs (1, 3, 4). Several studies have determined that peak vertical force and the associated stresses on the internal structures of the distal portion of the limb are higher in the forelimbs, leading to the suggestion that limb load distribution may play an important role in the development of musculoskeletal disorders in the horse (5-8). The authors of an *in vivo* study have proposed that biomechanical loading of tendons is involved in the initial enlargement of lesions (9). It is therefore conceivable that the differences in the load distribution and kinematics between fore- and hindlimbs might elicit different responses to tendon lesions in terms of lesion development, propagation and healing.

Several experimental models of equine SDFT tendinopathy have been developed; these are aimed at gaining a better understanding of the tendon healing process through the study of the response to artificially created lesions (10–13). These models have also been used to determine the effect of different therapeutic approaches to

9.2 Figures

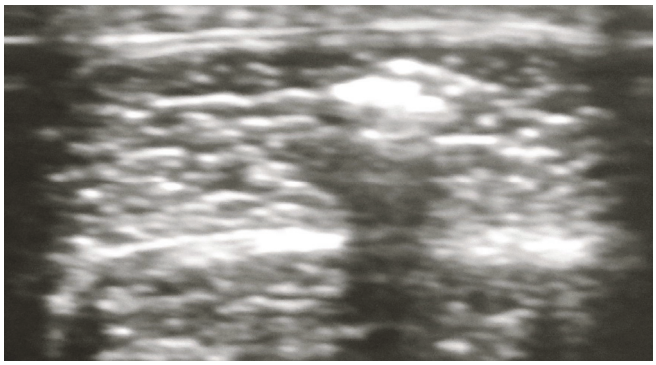
Figure 1. A) Surgical setting for the induction of superficial digital flexor tendon (SDFT) core lesions. An assistant extended the limb. The skin and paratendon were incised and the synovial resector was introduced into the core of the tendon in a proximal direction over a length of 7cm. Once in position, the synovial resector was activated and slowly retracted in approximately 20s while rotating the handpiece. B.1) The introduction of the synovial resector into the core of the SDFT was performed using ultrasonographic guidance. B.2) The acoustic shadow caused by the metallic synovial resector is visible in the core of the SDFT C) After each procedure an important amount of tendon fibers were retrieved in the tip of the synovial resector.

A)



B.1)

B.2)



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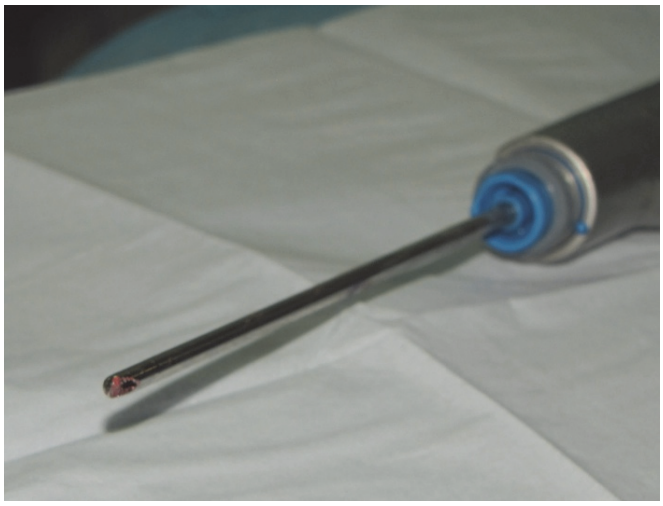


Figure 2. The tendons were injected using a 20G x 1½-inch needle. The procedure was performed on weight-bearing and the needle was introduced in a longitudinal plane in a plantaroproximal-dorsodistal direction, aiming at the proximal third of the lesions.

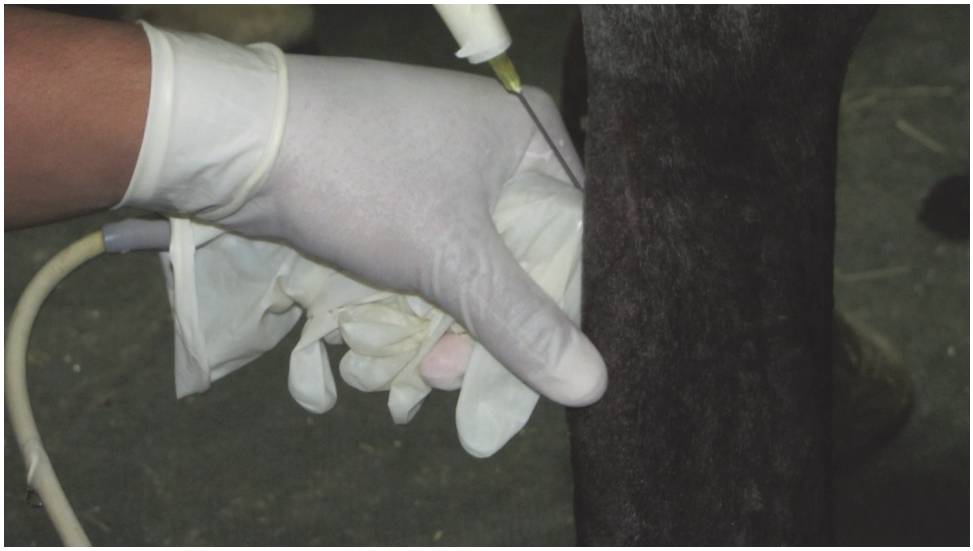


Figure 3. Setting for the ultrasonographic evaluation of the superficial digital flexor tendon. A measuring tape was positioned lateral to the limb. The limbs were divided in transverse and longitudinal zones as described in the literature and the distance distal to the accessory carpal bone or proximal to the calcaneal tuberosity was recorded for each zone. Re-examinations of every zone in each pair of fore- and hindlimbs were performed at the same distances.

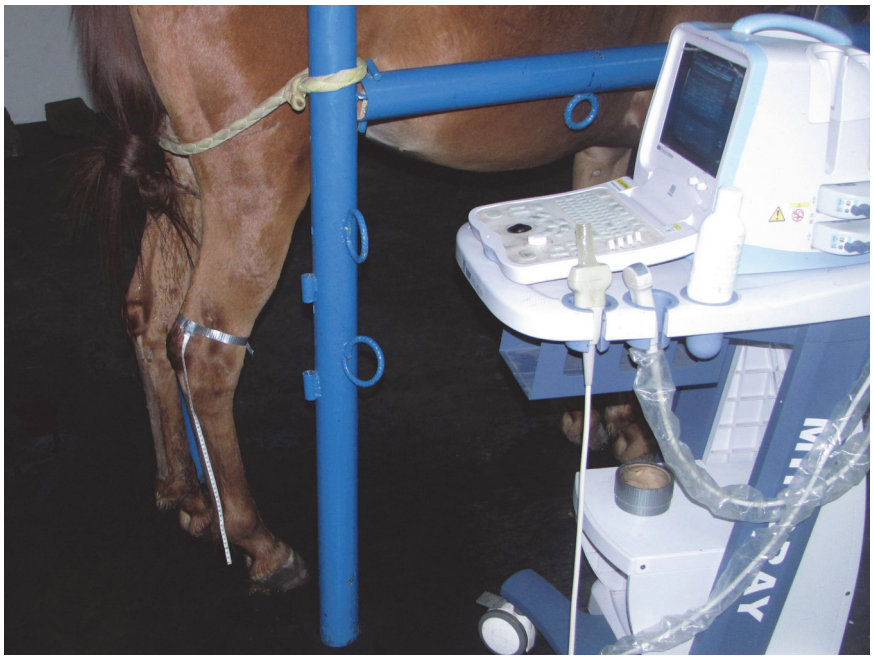
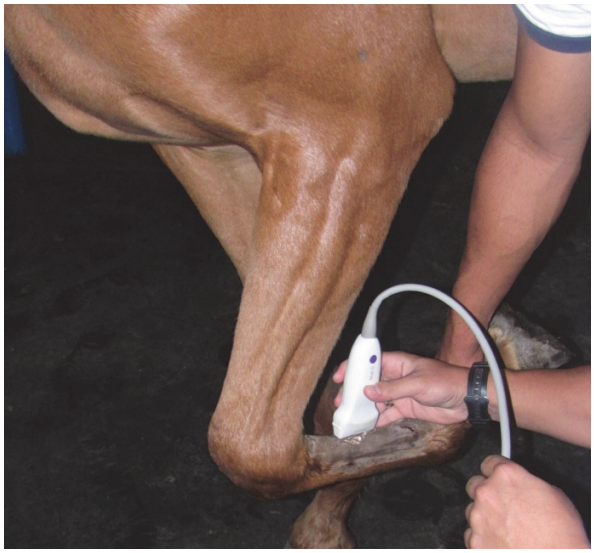


Figure 4. A) Color Doppler Ultrasound (CDU) evaluation was performed 24 weeks after lesion induction. The limbs were scanned longitudinally in semi-flexed position to relax the tendinous structures and therefore avoid the collapse of intra-tendinous vessels due to mechanical forces. B) The image sequences of the each scan were stored and the frame with the highest vascularization selected subjectively and then scored.

A)



B)

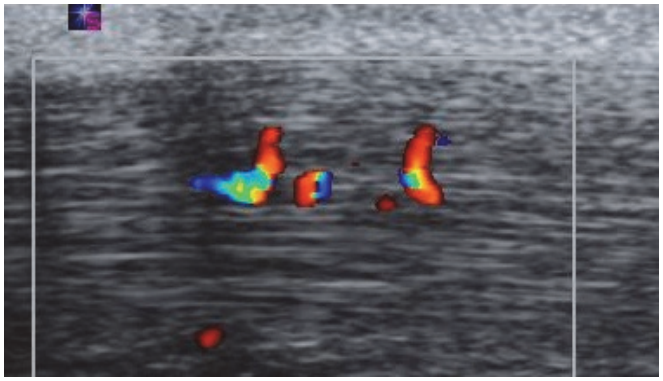
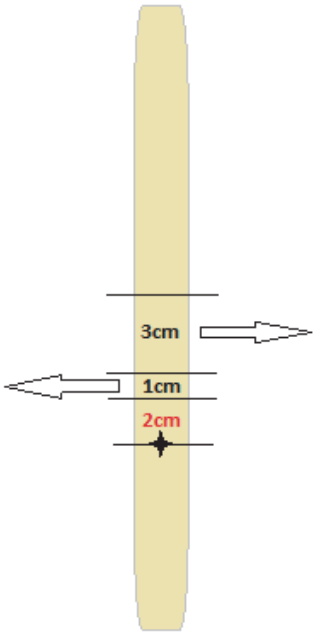
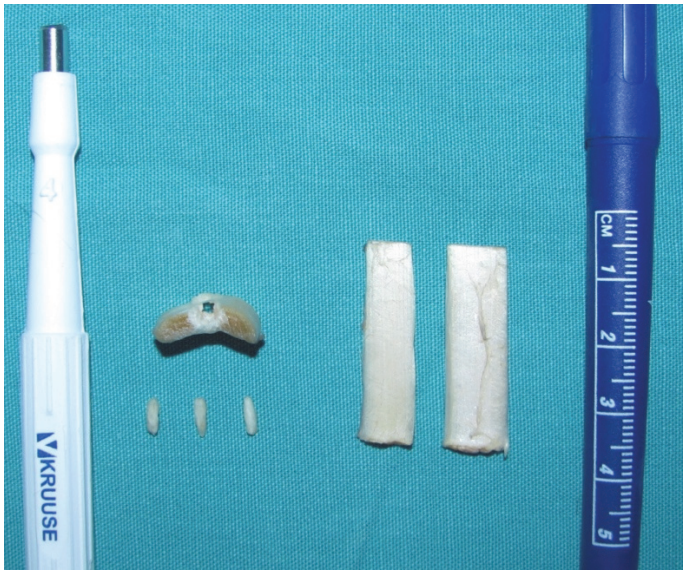


Figure 5. A and B) A transverse 1cm tendon slice was obtained 2cm proximal to the scar of the stab incision in the paratendon. The core lesions were identified and a 4mm punch directed longitudinally was used to harvest injured tissue only. These samples were divided in 3 sub-sections that were used to perform the biochemical analysis. Proximal to the aforementioned tendon slice, a 3cm segment was harvested and divided through the center of the lesion in two longitudinal sections that were used for biomechanical and histological evaluation.

A)



B)



X Oral Presentations

I. R.J. Estrada, P.R. Van Weeren, C.H.A. Van de Lest, J. Boere, M. Reyes, J.C. Ionita, M. Estrada, C.J. Lischer. **"**Comparison of healing in fore- and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon". Scientific Annual Meeting European College of Veterinary Surgeons (ECVS), Copenhagen, Denmark, July 2014.

XI List of publications

I. R.J. Estrada, P.R. Van Weeren, C.H.A. Van de Lest, J. Boere, M. Reyes, J.C. Ionita, M. Estrada, C.J. Lischer. "Effects of Autologous Conditioned Plasma ® (ACP) on the healing of surgically induced core lesions in equine superficial digital flexor tendon". Pferdeheilkunde, Nov. / Dec. 2014. 30 (6) (Accepted).

II. R.J. Estrada, P.R. Van Weeren, C.H.A. Van de Lest, J. Boere, M. Reyes, J.C. Ionita, M. Estrada, C.J. Lischer. "Comparison of healing in fore- and hindlimb surgically induced core lesions of the equine superficial digital flexor tendon". Veterinary and Comparative Orthopaedics and Traumatology. Jul 2014. 27(5).

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XIII Author´s declaration of originality

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 25. September 2014 Roberto J. Estrada, DVM