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Autologous platelet rich plasma for regeneration of tendon injuries in horses

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ABSTRACT

Digital flexor tendon injuries are the most common cause of early retirement and economic loss in the equine industry due to the poor healing tendency of the tendons. This study was conducted to improve the quality of tendon healing by using Autologous Platelet Rich Plasma incorporated collagen scaffold. Using ultrasound guidance, autologous Platelet Rich Plasma with and without collagen scaffold was engrafted intra-lesionally into 12 affected digital flexor tendons of 11 horses and healing was assessed periodically. Lameness score decreased in all the horses and the therapeutic outcome of intra-lesional engraftment of Autologous Platelet Rich Plasma with collagen scaffold was found to be superior in terms of clinical outcome, ultrasonographic and biochemical assessment. Ultrasonography served as an effective tool for diagnosis and for evaluation of healing tendon injuries.

Key words: Collagen type I, Equine, MMP13, Platelet rich plasma, Tendon injuries, Ultrasonography.

INTRODUCTION

Flexor tendon injuries in horses is the most common musculoskeletal problem causing early retirement from work and economic loss in the equine industry (Dowling *et al.*, 2000 and Dyson, 2004). The common structures affected are superficial digital flexor, deep digital flexor tendon and suspensory ligament. The popular Indian breeds of horses are Marwari and Kathiyawari known for their athletic performance also have the same incidence of these injuries. Tissue repair in musculoskeletal injuries is slow having long healing period which may take months for complete healing of the lesion because of poor blood supply which is one of the main challenges in the treatment of tendinitis. The current treatment of tendinitis like prolonged rest, blistering and intra lesional injection of corticosteroids with controlled exercise are not favorable and results in high recurrence rate (Goodship *et al.*, 1994).

Autologous Platelet Rich Plasma (PRP) therapy is an emerging technology that aims to improve the process of tissue repair through local delivery of autologous bioactive agents to influence critical physiological mechanisms such as inflammation, angiogenesis or extracellular matrix synthesis. Because of its autogenous origin, easy preparation, and excellent safety profile, the advent of PRP has opened another therapeutic door for muscle and skeletal regeneration (Filardo *et al.*, 2012).Combining with biomaterial scaffolds provide the structural support for cell attachment and subsequent tissue development. Collagen is widely used for biomedical and pharmaceutical application owing to its cell attachment capabilities, excellent biocompatibility, biodegradability and weak antigenicity (Caliari and Harley, 2011). These therapies are aimed at delivering the functional tissue equivalents of native tendon which can be regenerated using isolated Autologous Platelet Rich Plasma.

MATERIALS AND METHODS

The study included twelve limbs of eleven horses treated during the period from 2014-2015. The horses were subjected to lameness evaluation (graded as per grading system of American Association of Equine Practitioners) (Arguelles *et al.*, 2008), flexion test, diagnostic nerve block and ultrasonography to identify the site of lesion. Ultrasonography is preferred for assessment of soft tissue injuries.

Ultrasonographic examination: Horses were subjected to ultrasound examination, for identifying the exact location of lesion the metacarpal/ metatarsal region was divided into seven equal zones (Ia, Ib, IIa,IIb, IIIa, IIIb and IIIc) at 20 mm interval and 60 to 300m distal to the accessory carpal bone. The palmar/ plantar pastern region of the limb was divided into three zones P1, P2 and P3 (Fig 1 and 2). The ultrasonographic interpretation of tendon lesions was graded qualitatively and quantitatively into four types. The qualitative grading was based on the linearity of the tendon fibers (Caudry and Denoix, 2013).

Grade 1: > 75per cent of the fibers in the lesion are parallel
Grade 2: >50 per cent but ≤ 75per cent of the fibers in the lesion are parallel
Grade 3: > 25per cent but < 50per cent of the fibers are parallel
Grade 4: ≤ 25 per cent of the fibers are parallel

The quantitative scoring was based on the echogenicity of the lesion (Reef *et al.*, 1988).

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Type 1: Mild hypo-echogenicity with slight fiber alteration and leucocyte infiltration
Type 2: Presence of tendon hypo-echogenicity with fiber alteration
Type 3: Widespread hypo-echogenicity and fiber rupture
Type 4: Anechogenic tendon with complete fiber ruptures with haematoma.

Preparation of PRP: The platelet rich plasma was prepared by double centrifugation tube method (Perazzi *et al.*, 2013).Collagen hydrogel was extracted from fish scales by Acid Solubility Method (Zhang *et al.*, 2011). 2 to 5 milliliter of autologous platelet rich plasma depending upon the concentration of platelet count of 0.428 to 1.01x10^6 plt/µl (Giusti *et al.*, 2014) was used as the dose irrespective of the size of the horse (Table 1).

Twelve limbs were divided into two groups, Group I was injected with 2 to 5 milliliter of Autologous Platelet Rich Plasma per lesion to obtain a threefold increase from the base line platelet count. Horses in the Group II were injected with a suspension of 2 to 5 millilitre of Autologous Platelet Rich Plasma and 1 milliliter Collagen Hydrogel at the concentration of 6 mg/millilitre.

Engraftment and rehabilitational protocol: Either under standing sedation or regional analgesia, a 18 gauge needle was inserted through the skin perpendicular to the long axis of the tendon and autologous PRP or PRP with collagen hydrogel was slowly injected directly into the core lesion with the help of an ultrasound (Torricelli *et al.*, 2011 and Waselau *et al.*, 2008) (Fig 6). A sterile modified Robert Jhones bandage was applied to prevent leakage of interstitial fluid from the blood vessels for five days (Henninger, 1994). The horses were given complete rest for first two days. From third day, the horses were subjected to a controlled rehabilitation protocol that includes controlled walk by increasing the duration from 5 to 40 minutes each week and later trot was added every week until 30 minutes at the 25th week (Renzi *et al.*, 2013). No pain management was given to the horses during the rehabilitation period as they will suppress the cell migration, proliferation and remodeling of native cells (Halpen *et al*., 2012).

Table 1: Platelet concentration and Dosage.

Group	Horse No	Blood Collected (ml)	Platelet Concentration in Whole Blood	Platelet Concentration in First Centrifuge (Platelets/microlitre)	Platelet concentration in Second Centrifuge (PRP)(Platelets/ microlitre)	Platelet increase over baseline	Dose in ml
Autolgous PRP	1	15	2,42,000	5,04,000	8,01,000	2.3 fold	3ml
	2	15	3,00,000	6,73,000	10,01,000	2.3 fold	3.5ml
	3	15	1,84,000	3,91,000	6,52,000	2.5fold	3.2ml
	4	15	1,68,000	4,82,000	7,11,000	3.2 fold	2.8ml
	5	15	1,24,000	5,43,000	6,92,000	4.5 fold	3.8ml
	6	15	2,01,000	3,62,000	4,51,000	1.2 fold	3ml
Autologous PRP+ collagen	7	15	1,97,000	3,55,000	5,71,000	1.8 fold	5.2ml
	8	15	1,79,000	4,25,000	7,31,000	3 fold	4.7ml
	9	15	150,000	3,33,000	4,28,000	1.8 fold	5.5ml
	10	15	2,19,000	5,20,000	7,45,000	2 fold	4.6ml
	11	15	2,21,000	4,81,000	6,31,000	1.8 fold	5ml
	12	15	2,23,000	5,26,000	9,56,000	3.2 fold	4.8ml

Table 2: Mean serum ALP (IU/dL), CK (mmol/dL) and MMP13 (ng/ml) level in Group I and Group II s.

Biochemical parameter	Group	Pre-operative	One Week post - operatively	Four weeks post operatively	Eight weeks post operatively	F Value	P Value
ALP (IU/dL)	I	449.500±24.871^{c}	409.500±24.231^{bc}	403.333±33.467^{ab}	310.333±23.811^{a}	6.691	0.003**
	II	644.667±100.672^{b}	477.833±70.265^{ab}	392.167±28.203^{ab}	350.166±30.347^{a}	2.663	0.076
CK (mmol/dL)	I	16.333±3.402^{b}	14.667±3.252^{b}	11.333±2.333^{ab}	6.000±1.390^{a}	2.813	0.066
	II	17.167±3.618^{b}	15.500±3.373^{b}	10.833±2.587^{ab}	6.333±1.115^{a}	2.944	0.058
MMP13 (ng/ml)	I	305.000±53.119^{b}	261.333±49.130^{b}	182.333±36.641^{ab}	86.833±26.129^{a}	5.067	0.009**
	II	441.667±77.992^{c}	401.667±72.900^{bc}	253.500±45.973^{ab}	97.000±25.409^{a}	6.948	0.002**

*Significant, ** Highly Significant, 'a' represents significant difference within a group and 'b' represents significance between groups, 'c' represents significant difference among the groups.

The horses were examined clinically by scoring the degree of lameness and flexion test, ultrasonographic examination and the level of serum ALP, MMP13 and CK in the 1st week, 4th week, 8th week and 6 months later. The statistics was carried out using non-parmetric test Mann-Whiteny and Kruskal Wallis for the scores and two-way ANOVA using the software SPSS 17.

RESULTS AND DISCUSSION

The lameness and flexion test score decreased from pre-injection day to eight weeks post operative in both the groups. Ultrasonographycally, location of lesion in the tendon was distributed among four zones which includes lesion in zone1 (1a and 1b), 3 lesions in zone2 (2a and 2b), 6 lesions in zone 3 (3a, 3b and 3c) of metacarpus/metatarsus and 3 lesions in zone P (P1, P2 and P3) of pastern region. The distribution was found to be more in zone 3. Qualitative ultrasound analysis showed that more than 25% fiber alignment was noticed 8 weeks post operatively in both the groups but in Group II healing was found to be more homogenous and uniform. Statistical analysis revealed that there is no significant ($p>0.05$) difference in the distribution of ultrasonographic score between the groups. In Group I, there is significant ($p<0.05$) decrease in ultrasonographic score between the periods pre-injection day and eight weeks, one week and eight weeks and pre-injection and four weeks (Fig 3). In Group II, there is significant ($p<0.05$) decrease in distribution of ultrasonographic score between the periods pre-injection day and eight weeks and pre-injection day and four weeks (Fig 4 and 5). Also there was a significant decrease in concentration of serum alkaline phosphatase, creatinine kinase and MMP13 between the period pre operative day to eight weeks in both the groups (Table 2).

Fig 1: Ultrasonographic division of metac arpal region.

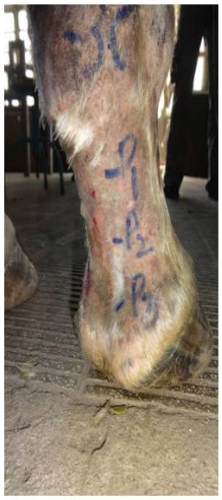


Fig 2: Ultrasonographic division of palmer aspect of pastern region.

The poor healing tendency of tendon has been attributed to the high ECM-cell ratio (Goodship *et al*., 1994). Hence the Autologous Platelet rich Plasma is used for the treatment of tendinitis to increase the healing potential of tendon which enhances the fibroblast proliferation. The distribution of lesion in the ultrasonographic zones were found to be more in the zone 3 near the manica flexoria which was a thin synovial and fibrous fold that attaches to the both side of SDFT (Caudry and Denoix, 2013). The

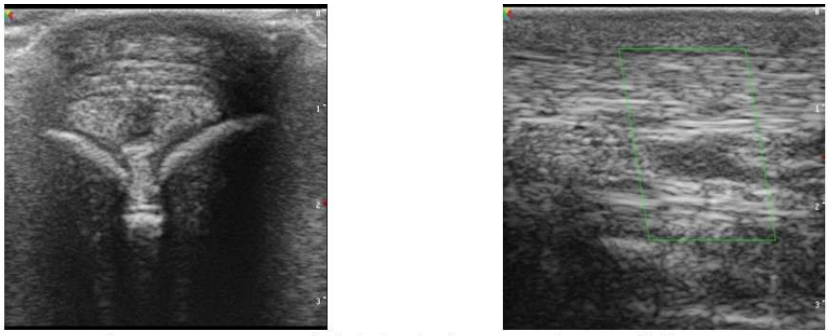
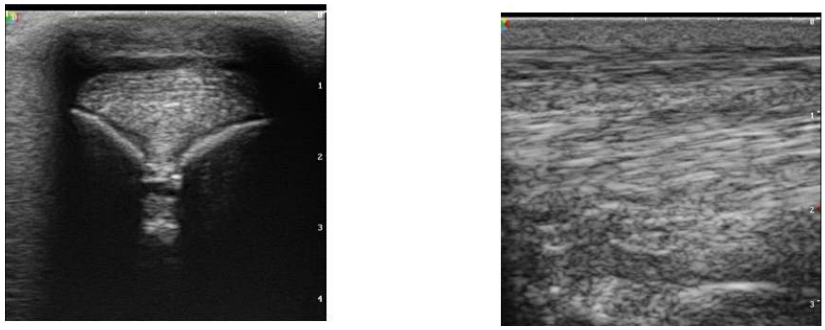


Fig 3: Group I-Anechoic lesion in the center of DDft - 0th day



8 weeks post pperatively-Increase in echogenicity at the lesion site.

Platelet Rich Plasma when activated releases various growth factors like transforming growth factor β (TGF beta), insulin like growth factors, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factors (EGF) (Anitua, 1999 and Maia *et al.*, 2009). Plate Rich Plasma could increase both the number of cells and the cellular component by enhancing fibroblast proliferation and collagen production, induces differentiation of Tendon stem cell/ Progenitor cell into tenocytes, increases the migration of circulation-derived stem cells, which are more proliferative and produce more collagen type I (Young *et al.*, 1998). The insulin like growth factor plays a critical role in enhancing muscle regeneration after injury by stimulating myoblast proliferation and differentiation (Engert *et al.*, 1996). The type I collagen is predominantly present in tendon (Dowling *et al.*, 2000 and Smith and Goodship 2004). During normal healing process the infiltrating fibroblast appear morphologically different from normal tendon fibroblast (Obaid and Connell, 2010). The scar tissue that is formed after healing is of type III collagen which is inferior in strength to type I collagen. The PRP rebalance the healing equation and move towards the formation of type I collagen rather than type III collagen (Zhang *et al.*, 2011 and Majewsk *et al.*, 2009). Thus the healing is enhanced in Autologous Platelet Rich Plasma engraftment. Hence Platelet Rich Plasma which is a higher concentrate of platelet would eventually secrete higher level of growth factors on intra-lesional injection in the affected tendon that would enhance the healing process of the tendon.

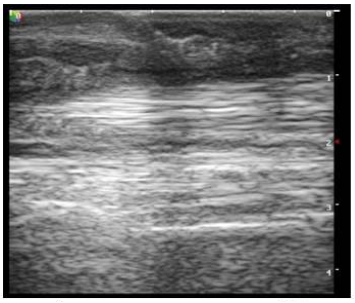
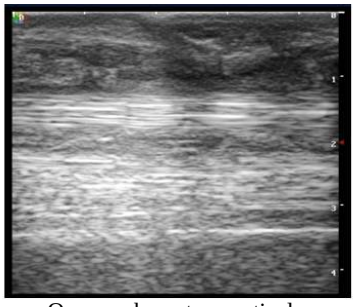
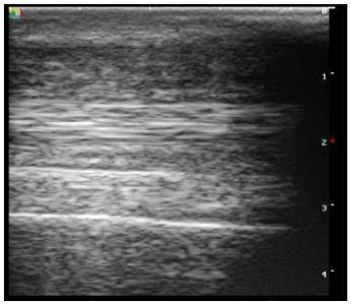


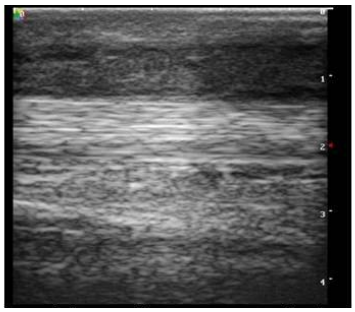
Fig 4: Group II: Partial laceration of SDFT-0th day.



One week post operatively



Four weeks-Post operatively-Increase in linearity fibers



Eight weeks post operatively-Uniformly healed fibers

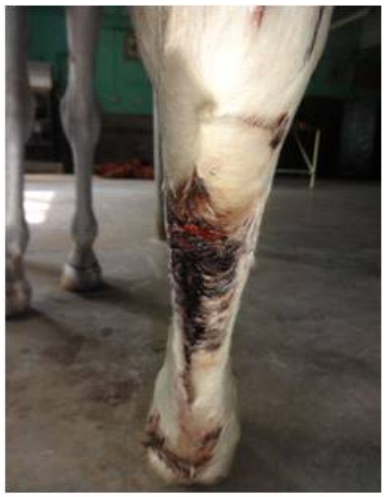


Fig 5: Laceration of tendon SDFT Preoperative.

Healwound-8 weeks post operative

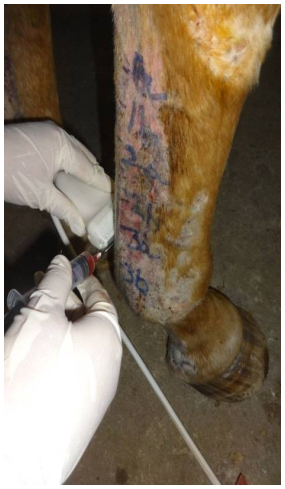


Fig 6: Ultrasound guided intra-lesional injection of PRP with collagen.

Platelet Rich Plasma at a concentration of 0.5 to 1x 10^6plt/µl was found to be ideal for tenocyte behavior (Giusti *et al.*, 2014). A lower concentration of 2.5 times the baseline levels was optimal to induce cell proliferation of osteoblasts and fibroblasts, while higher concentrations resulting in reduced numbers of osteoblasts and fibroblasts (Graziani *et al.*, 2006). The leukocyte count was kept lower than 5x10^5plt/µl in the present study as higher concentration of leukocyte was found to induce release of inflammatory cytokines (Cross *et al*., 2014 and Mishra and Pavelko 2006).

In Autologous Platelet Rich Plasma and collagen hydrogel group, an apparent decrease in ultrasonographic score, increase in echogenicity and organization of tendon fibers was noticed compared to Autologous Platelet Rich Plasma group which could be attributed to the combined effect of Autologous Platelet Rich Plasma and collagen scaffold that improved the ultrasonographic score as the collagen contributed to improvement in fiber aligmnent and echogenicity of the lesion. Collagen type 1 act as scaffolding material that mimic the native physiology of tendon, there for tissue by enhancing tendon cell motility, viability and metabolic activity (Caliari and Harley, 2011), this brings about mechanical changes like increase in strength, texture and elasticity of tendon for tissue regeneration (Bareil *et al.*, 2010). A liquid or pre-gel form of collagen can further expand the clinical utility of an ECM scaffold by allowing the delivery of the Platelet Rich Plasma via minimally invasive methods to sites of lesion (Badylak *et al.*, 2009). Collagen activated Platelet Rich Plasma resulted in a slower and sustained release of growth factors over 10 days. Platelet Rich Plasma activated with collagen caused less reduction of Platelet Rich Plasma clot over 50 percent (Harrison *et al.*, 2011). In similar studies done by Young *et al.*, (1998) and Awad *et al.*, (2000) who had seeded stem cells in collagen hydrogel for treatment of tendinitis found that the cells reoriented and expanded significantly with time. Bashandy *et al.*, (2014) and Punduk *et al.*, (2014) noticed a significant elevated activity of serum Alkaline Phosphatase and Creatinine kinase in most of the common musculoskeletal affections and post exercise in horses. An apparent decrease in serum Creatinine kinase level in Autologous Platelet Rich Plasma and collagen hydrogel group was noticed due to the sustained action of Autologous Platelet Rich Plasma in the collagen scaffold environment (Harrison *et al.*, 2011 and Punduk *et al.*, 2014).

MMP13 levels were assessed to evaluate the extent of extracellular matrix damage (Clegg et al., 2007 and Riley *et al.*, 2002). The serum MMP13 was determined using Horse MMP13 ELISA kit (CUSABIO CSB-EL014660HO) in this study (Bedi *et al.*, 2010, Castagna *et al.*, 2013 and Gao *et al.*, 2012). There was a significant decrease in serum MMP13 in both the groups from pre injection period to eight weeks post operatively and an apparent decrease in serum MMP13 level in Autologous Platelet Rich Plasma and collagen hydrogel group was noticed due to the sustained action of Autologous Platelet Rich Plasma in the collagen scaffold environment. This is due to the action of growth factors TGF β-1 and IGF-1 which upregulates the level of tissue inhibitors of matrix metalloproteases (Cross *et al*., 2014 and Harrison *et al.*, 2011).

No delayed healing was encountered in the present study in any of the horses during the study period. This could be attributed to regeneration of the tendon due to the action of Autologous Platelet Rich Plasma and collagen scaffold. Activated PRP acts not only as a native carrier of multiple growth factors, which stimulate cell proliferation, but also as a 3-dimensional bioactive scaffold (fibrin gel) with a mesh-like microstructure, which enhances cell migration and proliferation (Yuan *et al*., 2013). These factors with the sustained action of autologous Platelet Rich Plasma contributed to the enhanced healing of tendon and early return of horses to work.

CONCLUSION

To conclude, Autologous Platelet Rich Plasma with and without collagen scaffold after intra-lesional engraftment for tendinitis of digital flexor tendons in horses was found to be a novel and effective treatment modality as demonstrated by lameness score, flexion test score, ultrasonographic and biochemical evaluations. The therapeutic outcome of intra-lesional engraftment of Autologous Platelet Rich Plasma with collagen scaffold was found to be superior in terms of clinical outcome, ultrasonographic and biochemical assessment which could be due to the regeneration of the affected digital flexor tendon. Also ultrasonography served as an effective tool for diagnosis of tendinitis and also for evaluation of tendon healing.

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