Autologous platelet rich plasma for regeneration of tendon injuries in horses

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Received: 05-06-2018 Accepted: 21-01-2019 DOI: 10.18805/ijar.B-3653

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 300mm 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 echogenicity of the lesion (Reef et al., 1988).
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 6mg/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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Horse No</th>
<th>Blood Collected (ml)</th>
<th>Platelet Concentration in Whole Blood</th>
<th>Platelet Concentration in First Centrifuge (Platelets/microlitre)</th>
<th>Platelet Concentration in Second Centrifuge (PRP)(Platelets/microlitre)</th>
<th>Platelet increase over baseline</th>
<th>Dose in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous PRP</td>
<td>1</td>
<td>15</td>
<td>2.42,000</td>
<td>5.04,000</td>
<td>8.01,000</td>
<td>2.3 fold</td>
<td>3ml</td>
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<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>3.00,000</td>
<td>6.73,000</td>
<td>10.01,000</td>
<td>2.3 fold</td>
<td>3.5ml</td>
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<tr>
<td></td>
<td>3</td>
<td>15</td>
<td>1.84,000</td>
<td>3.91,000</td>
<td>6.52,000</td>
<td>2.5 fold</td>
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<td>4</td>
<td>15</td>
<td>1.68,000</td>
<td>4.82,000</td>
<td>7.11,000</td>
<td>3.2 fold</td>
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<tr>
<td></td>
<td>5</td>
<td>15</td>
<td>1.24,000</td>
<td>5.43,000</td>
<td>6.92,000</td>
<td>4.5 fold</td>
<td>3.8ml</td>
</tr>
<tr>
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<td>6</td>
<td>15</td>
<td>2.01,000</td>
<td>3.62,000</td>
<td>4.51,000</td>
<td>1.2 fold</td>
<td>3ml</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>15</td>
<td>1.97,000</td>
<td>3.55,000</td>
<td>5.71,000</td>
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<td>8</td>
<td>15</td>
<td>1.79,000</td>
<td>4.25,000</td>
<td>7.31,000</td>
<td>3 fold</td>
<td>4.7ml</td>
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<td>9</td>
<td>15</td>
<td>150,000</td>
<td>3.33,000</td>
<td>4.28,000</td>
<td>1.8 fold</td>
<td>5.5ml</td>
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<tr>
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<td>2.19,000</td>
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<td>15</td>
<td>2.21,000</td>
<td>4.81,000</td>
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<td>5ml</td>
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<td>15</td>
<td>2.23,000</td>
<td>5.26,000</td>
<td>9.56,000</td>
<td>3.2 fold</td>
<td>4.8ml</td>
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</tbody>
</table>

Table 2: Mean serum ALP (IU/dL), CK (mmol/dL) and MMP-13 (ng/ml) level in Group I and Group II.

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Group</th>
<th>Pre-operative</th>
<th>One Week post operatively</th>
<th>Four weeks post operatively</th>
<th>Eight weeks post operatively</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (IU/dL)</td>
<td>I</td>
<td>449.500±24.871</td>
<td>409.500±24.231</td>
<td>403.333±33.467</td>
<td>310.333±23.811</td>
<td>6.691</td>
<td>0.003**</td>
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<tr>
<td></td>
<td>II</td>
<td>644.667±100.672</td>
<td>477.833±70.265</td>
<td>392.167±28.203</td>
<td>350.166±30.347</td>
<td>2.663</td>
<td>0.076</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>17.167±3.618</td>
<td>15.500±3.373</td>
<td>10.833±2.587</td>
<td>6.333±1.115</td>
<td>2.944</td>
<td>0.058</td>
</tr>
<tr>
<td>MMP-13 (ng/ml)</td>
<td>I</td>
<td>305.000±53.119</td>
<td>261.333±49.130</td>
<td>182.333±36.641</td>
<td>86.833±26.129</td>
<td>5.067</td>
<td>0.009**</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>441.667±77.992</td>
<td>401.667±72.900</td>
<td>253.500±45.973</td>
<td>97.000±25.409</td>
<td>6.948</td>
<td>0.002**</td>
</tr>
</tbody>
</table>

*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-parametric test Mann-Whitney 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. Ultrasonographically, location of lesion in the tendon was distributed among four zones which includes lesion in zone 1 (1a and 1b), 3 lesions in zone 2 (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).

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).
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 intralenseral 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
Platelet Rich Plasma at a concentration of 0.5 to 1x 10^6 plt/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 5x10^6 plt/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 alignment and echogenicity of the lesion. Collagen type 1 act as scaffolding material that mimics the native physiology of tendon, therefore 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., (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 Panduk 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 Panduk 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 TGFβ-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.
REFERENCES


