TITLE: Regenerative medicine in equine orthopaedics: what and when?

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Regenerative medicine in equine orthopaedics: what and when?

Abstract

The development of regenerative medicine has opened up many new therapeutic avenues in veterinary medicine. The focus of regenerative medicine in the horse lies primarily in the musculoskeletal system, where the consequences of injury make tendons, ligaments and joints particularly desirable targets for such interventions. This article focuses on what has been learned from the use of regenerative medicine in naturally-occurring tendon, ligament and joint disease in the horse.

Introduction

Defining regenerative medicine is difficult because the name implies the ability of the treatment to regenerate damaged tissues and, while this remains the goal, there is little evidence that any of the currently available treatments actually achieve this. Therefore the term 'biological therapy' is probably more suitable than regenerative medicine. However, this does not mean that the treatments are ineffective and there is an accumulating robust body of evidence, certainly for mesenchymal stem cells (MSCs), that they are able to modulate inflammation via paracrine activity and thereby induce better quality healing than natural repair.

The consequences of injury within the musculoskeletal system (altered function because of fibrous healing) and the physical features of the disease (a 'receptacle' assisting ease of administration intralesionally) make tendon a particularly desirable target for the use of biological therapies. Other musculoskeletal diseases where regenerative medicine has attracted interest are in the treatment of joint disease, while work involving the use of veterinary clinical cases, mainly in small animal, have also investigated its use in spinal cord trauma and cardiac disease.

There has been a considerable amount of work in experimental animal models on the effects of regenerative medicine on tendon healing and other musculoskeletal diseases. While these models serve an important role in investigating efficacy and mechanisms of action, they do not completely replicate all the features of natural disease. Therefore, studying the effects of these treatments in clinical cases provides more

relevant information for the clinician and has taught us a considerable amount about this technology.

Regenerative medicine generally encompasses the use of one or more of three components: scaffolds, growth factors, and cells. This article focuses on what has been learned from the use of the three most commonly used biological therapies: platelet-rich plasma (PRP), interleukin-receptor antagonist protein (IRAP) and mesenchymal stem cells (MSCs), in naturally-occurring tendon, ligament and joint disease in the horse.

Platelet-rich plasma

Platelet-rich plasma/concentrate (PRP) has gained popularity in the treatment of tendon and ligament injuries and, more recently, joint disease. Platelet-rich plasma contains high levels of those growth factors sequestered in platelets — most notably platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-beta1), but also vascular endothelial growth factor (VEGF), which promotes neovascularisation.

It is not known whether these growth factors are optimal for tendon and ligament healing, but they have been demonstrated to have anabolic effects in vitro on both tendon explants from the superficial digital flexor tendon (SDFT) (Schnabel et al, 2007) and suspensory ligament explants and desmocytes (Smith et al, 2006; Schnabel et al, 2008), and are therefore logical factors to consider.

However, because of the close association of platelets with normal fibrous healing, it could be postulated that PRP would promote an exaggerated fibrotic reaction rather than regeneration. Studies in experimental mechanically created tendon lesions in the horse have demonstrated improved organisation and in-creased strength after PRP treatment, although the stiffness of the repairing tissue was also increased (Bosch et al, 2010). Use of PRP should therefore be best considered for those indications where exaggerated fibrosis is potentially less of a concern or even desired as part of the therapeutic strategy, such as to encourage the healing of a persistent lesion or after a severe mechanically destabilising injury that requires as rapid repair as possible. In addition, PRP is highly suitable for intra-operative use of biological therapies — which necessitates a 'horse-side' preparation.

A further concern has been raised with regards to the presence of high white blood cell counts in some PRP preparations, because of the possibility that these cells will

induce further inflammation through the release of inflammatory cytokines. It is not clear whether this concern has any relevance to the clinical use, but the use of PRP preparations with high white cell counts has been associated with increased inflammatory signs in joints after administration (Smit et al, 2019), therefore low white cell count preparations might be better to use when injecting intra-synovially. No reactions has been reported following injection of PRP with high white blood cell counts into soft tissues.

Platelet-rich plasma can be prepared by either centrifugation or filtration, but is highly variable between individuals and preparatory techniques (Hessel et al, 2015). A closed filtration system has been developed (V-PET; Pall Corporation) that can be carried out horse-side and has the advantage of not requiring additional equipment, which minimises sample handling and therefore the risk of iatrogenic contamination. The technique involves taking 55 ml of venous blood, which is mixed with a capture solution in the collection bag before being passed through a filter where the platelets are captured. Back-flushing the harvest solution through the column recovers the platelet concentrate into a syringe with an average volume of 6 ml.

Evidence of efficacy in comparison to other conventional treatments or natural repair is still lacking, although recent publications have suggested positive effects in small clinical series of both suspensory ligament and SDFT injuries (Arguelles et al, 2008; Waselau et al, 2008). In a series of clinical cases treated with the filtered platelet concentrate, the technique has been very well tolerated with no evidence of clinical inflammation after treatment, and has demonstrated resolution of the lameness and the ultrasonographic lesions within 2 months (Castelijns et al, 2011).

There are no large data sets published on the efficacy of PRP in joints. Anecdotal reports suggest that the use of PRP in equine joints is safe and potentially beneficial, and a study suggests that it is effective in 50% of cases with osteoarthritis (Mirza et al, 2016). Dosages for intra-articular administration are similar to those for IRAP (see later).

A survey of 123 studies on the use of PRP in equine and human musculoskeletal lesions reported that just under 50% of the studies reporting a beneficial effect. However, two-thirds of the studies that showed a beneficial effect had a high level of potential bias, while two-thirds of the studies that reported no benefit had a low risk of bias (Brossi et

al, 2015). Recently, a well-powered study for Achilles tendon rupture in humans showed no benefit of PRP (Keene et al, 2019).

Interleukin-1 receptor antagonist protein (IRAP; Orthokine; Autologous Conditioned Serum (ACS))

Interleukin-1β (IL-1β) is one of the most prominent mediators at the top of the cascade for cartilage degeneration for which there is a naturally occurring inhibitor of IL-1, interleukin receptor antagonist protein (IRAP) which binds to the IL-1 receptor to block the action of IL-1β. This molecule is therefore an ideal candidate as a disease-modifying osteoarthritic drug (DMOAD) as opposed to the current osteoarthritic drugs which are symptom-modifying (SMOADs). Proof of concept for its effectiveness has been shown in a arthritis model using gene transfection (Frisbie et al, 2002).

Incubation of peripheral blood over 24-hour at 37C results in stimulation of monocytes to produce an autologous conditioned serum (ACS) that contains elevated levels of:

- interleukin-1 receptor antagonist protein
- other beneficial cytokines and growth factors (Meijer et al, 2003).

The incubated syringe can then be centrifuged to obtain the serum for injection into the affected joints. A 50ml syringe of blood usually yields between 20-25ml of ACS. This is the equivalent of 5-6 doses (see Table 1 for suggested dosages) which can be stored frozen for subsequent use. The duration of IRAP in the joint, however, has been shown to be short – levels have been shown to return to baseline between 4 and 48 hours after injection.

When tested in an equine OA model, injection of 6 mL ACS on days 14, 21, 28, and 35 resulted in:

- significant improvement in the degree of lameness on day 70
- significant decrease in synovial membrane hyperplasia
- significant increase in synovial fluid concentration of IRAP (Frisbie et al, 2007).

The original indication was joint disease that failed to respond to intra-articular corticosteroids. However, clinical impressions of its use in chronic lameness have been disappointing. Most commonly it appears to be used mainly as ‘maintenance’ injections in mild lameness and performance problems in competition horses. However, a

controlled study in naturally-occurring osteoarthritis in horses showed a significant benefit in lameness using a similar, but white-cell rich, commercial preparation (APS; Prostride™)(Bertone et al, 2014). IRAP has also been investigated in the treatment off SDFT injuries where it showed potentially some subtle benefits (Geburek et al, 2015).

Mesenchymal stem cells

Choice of cell and choice of commercial products

The choice of cells has been more influenced by commercial availability and legislation than necessarily knowing which are the most effective, as few scientific comparisons have been performed to date. The two most common products available differ more by the preparation method than cellular source — these are the 'minimally manipulated' cell preparations from adipose tissue and the cultured preparations from bone marrow (Figures 1 and 2). The adipose preparations allow horse-side treatments that are more straight-forward and possibly easier to obtain licensing for, while the cultured bone marrow-derived MSCs are more homogenous but the technique more demanding (a two-stage process involving aspirating bone marrow from the sternum or tuber coxae followed by ex-vivo culture). In addition, there are other sources being used clinically, including cells recovered from peripheral blood, umbilical cord and teeth. Until recently, most clinically-used treatments were autologous, but there has been enthusiasm to adopt allogenic treatments because they are potentially cheaper and easier to undertake. However, legislation can limit their use because of logical additional safety concerns. So far, there have been few serious adverse reactions seen with the allogenic cells, although there have been detectable reactions when the implanted cells are MHC Class II positive (Schnabel et al, 2014) and with repeated injections (Ardanaz et al, 2016). Even though obvious adverse reactions may not be evident, there may be increased clearance of allogenic cells before they are able to exert their therapeutic effects, although initial retention rates are similar (see later). This indicates the need for better characterisation for clinical products and strategies for evading the host immune response (such as encapsulation). There are now good cell surface markers for equine cells (e.g. CD90, CD29 (de Mattos Carvalho et al, 2009; Ranera et al, 2011; Paebst et al, 2014)), which offer easier characterisation compared to the traditional trilineage assays (differentiation into cartilage, bone or fat) as these are time-consuming, poorly

quantifiable and may lack relevance because they do not include differentiate into the target tissue (eg, tendon) and may not reflect the true mechanism of action.

Treatment protocol modifications

For cultured cells, correct transport is essential to maintain cell viability. Implantation should be within 24 hours to minimise cell death, while longer transport intervals benefit from freezing (Garvican et al, 2014a). Post intra-lesional injection, needle tracts are often visible and, while they have not seemed to alter outcome, there has been a tendency to use smaller diameter needles as a strategy to prevent them. However, needle size can influence the implanted stem cells by inducing apoptosis (Garvican et al, 2014a), therefore a minimum gauge of 20G is recommended (Garvican et al, 2014a). In addition, concurrent administration of MSCs with corticosteroids or certain antibiotics is discouraged because of their effects on cell viability (Parker et al, 2012; Bohannon et al 2013; Edmonds et al, 2017).

Tendon and ligament injury

a) *Extra-thecal tendon disease*

Injuries to tendons outside a tendon sheath (eg superficial digital flexor tendinopathy) heal by fibrosis and the resulting tissue is functionally deficient compared to normal tendon. This has important consequences for the animal in terms of altered limb mechanics, reduced performance and a substantial risk of re-injury. Since the original publication demonstrating the feasibility of treating equine tendon injuries with MSCs (Smith, et al, 2003), the Tendon Biology Group of the RVC have investigated, both experimentally and clinically, the efficacy of bone marrow-derived MSCs (BM-MSCs) implanted under ultrasound-guidance into naturally-occurring superficial digital flexor tendinopathy.

Tissue effects

A controlled experimental study of naturally-occurring SDFT injuries (Smith et al, 2013) demonstrated that BM-MSC treatment 'normalised' many tissue parameters so that they were closer to the contralateral, relatively normal and untreated, tendons than saline-injected controls.

Engraftment and survival

Initial studies in experimental equine models suggested there was only limited cell retention after implantation (Guest et al, 2010) and so we have investigated this further in naturally-occurring superficial digital flexor tendinopathy. Labelled mesenchymal stem cells showed survival for up to 4 months in limited numbers, where the distribution was largely within the endotenon (Y. Kasashima – unpublished data; Figure 3). No cells were retained when implanted into open defects. Non-invasive monitoring using Tc^{99}-HMPAO showed only 24% retention after 24 hours but this was still considerably more than when implanted by regional perfusion or intravenously (Becerra et al, 2013). As a strategy to increase the number of retained cells, we (at the RVC) have increased the 'standard' dose from 10 to 20 million and this has been supported by clinical outcome data (see below). Work is underway to enhance retention by using scaffolds (solid or injectable). Larger lesions are also treated with greater numbers of cells. In addition, the more limited spread seen in these studies has encouraged implantation at transition zones and the use of longitudinal injection technique to improve distribution of the cells.

Clinical effectiveness

An adequately powered study to demonstrate clinically meaningful reductions in the re-injury rate for superficial digital flexor tendinopathy requires treatment group sizes of approximately 100 horses (Smith and McIlwraith, 2012). An independently analysed study with n=113 evaluated the clinical outcome of SDFT injuries treated using the same technique in the UK (Godwin et al, 2012). Ultrasonographic appraisal of treated cases show a rapid filling-in of the hypoechoic lesions, although the longitudinal striated pattern did not return to normal. Histopathological examination on 17 tendons from postmortem samples obtained from 12 horses that had undergone MSC implantation showed both good quality healing with minimal inflammatory cells and crimped, organised collagen fibres. Furthermore, there was no evidence of any abnormal tissue or neoplastic transformation. In racehorses, the re-injury percentage was 27%, which was a significantly reduced re-injury rate (approximately one-half) compared with two other studies in racehorses where the inclusion and follow-up criteria were identical ($p<0.05$ versus 56% (Dyson, 2004); $p<0.01$ versus 50% (O'Meara et al, 2010)). Outcome has also

been analysed for SDFT injuries in sports horses treated with MSCs by the VetCell company (not independently validated; n=68). The re-injury rate was 19%, which was also approximately one half that previously documented for sports horses (Dyson, 2004). The re-injury rates were lower with shorter interval between injury and implantation and with ≥20 million cells (supporting increasing the standard dose to 20 million cells).

b) Intra-thecal tendon disease

Intra-thecal tendon injuries (e.g. deep digital flexor tendon tears within the digital sheath) carry very different challenges. These lesions frequently communicate with the synovial cavity where synovial fluid causes death of the resident tenocytes (Garvican et al, 2017), and intra-thecal tendon has reduced cell numbers, no paratenon and limited vascularity, which limit healing. Mesenchymal stem cells coat the surface of tendon explants in vitro and modulate the release of matrix proteins (Garvican et al, 2014b), hence intra-thecal delivery of MSCs might offer a therapeutic benefit for an injury that is currently managed by debridement but carries a low success rate. However both an experimental study in sheep using both bone marrow-derived and synovium-derived MSCs (Khan et al, 2018; Khan et al, 2020), and a clinical trial in horses, failed to show any benefit in healing of tendon defects inside the digital sheath. Labelling of the cells showed that implanted MSCs engraft ed into the sheath synovium without any cells being present in the tendon defect (Khan et al, 2018; Khan et al, 2020), hence the intra-thecal injection of MSCs is currently not recommended for the treatment of intra-thecal tendon disease.

Joint disease

When tested in an equine OA model, injection of MSCs resulted in a significant reduction in PGE2 level with bone marrow derived MSCs and a significant increase in TNFalpha levels with fat de-rived MSCs, but with no other significant effects on other more clinically relevant parameters (Frisbie et al, 2009). While this early work suggested low efficacy, this has not prevented their wide-spread use with anecdotal reports of success, and more recent publications have suggested better clinical benefits (Frisbie et al, 2011; Broeckx et al, 2014). This recent work has led to the first cell product (Arti-Cell Forte, Boehringer Ingelheim) to gain European Medicine Agency market authorisation. This cell product consists of allogenic mesenchymal stem cells, recovered from equine blood and differentiated towards a chondrocytic phenotype where the cells lose their

immunomodulatory capacity. While this seems to be at odds to the current hypotheses for stem cell mechanism of action in clinical disease, clinical studies have suggested that they are effective and the product has recently entered clinical use in the UK for the treatment of osteoarthritis.

Intra-articularly administered MSCs do not appear to adhere well to cartilage and again mainly engraft into synovium. However, in an experimental menisectomy model in a large animal model (the goat), injected MSCs demonstrated both a reduction in severity of subsequent osteoarthritis and a degree of meniscal neogenesis not seen in controls (Murphy et al, 2003). Consequently there has been interest in using MSCs for the treatment of problematic meniscal injuries in horses, which carry a poor prognosis. A publication has supported this hypothesis, with improved effectiveness of MSCs in naturally-occurring meniscal injuries (Ferris et al, 2014) although the degree of improvement was mild. This author considers their use for the treatment of grade III meniscal tears, identified ultrasonographically or arthroscopically, where the joint is stable.

A third use in joints is for incorporation with a bone substitute for the filling of subchondral bone cysts arthroscopically. No clinical series have been published to date but anecdotal reports on clinical cases show that they can be effective but are probably best reserved for bone cysts that have been refractory to more conventional therapeutic approaches.

Conclusions

There is currently a wide array of stem cell preparations available to the practitioner. It is presently not clear which offers the most effective treatment, but better characterisation of biological therapies and comparative studies are desirable future goals to inform which are the best to use. Experimental and clinical evidence in naturally-occurring tendon injury has shown that MSCs can have a significant benefit in the treatment of over-strain injuries where there is a contained lesion, with the greatest effectiveness being for large lesions treated early. In contrast, the evidence for the efficacy of intra-synovially administered MSCs for tendon tears inside tendon sheaths and bursae, or for joint disease, is more limited, so strong conclusions about their efficacy can not

currently be made, although it is possible that they can offer some benefit for the treatment of some joint diseases.

Figure legends:

Figure 1 – Equine mesenchymal stem cells in culture. These cells can be recovered from a variety of mesenchymal tissues, the most commonly used being bone marrow and fat but blood, umbilical cord and teeth are other sources that have been developed for clinical use.

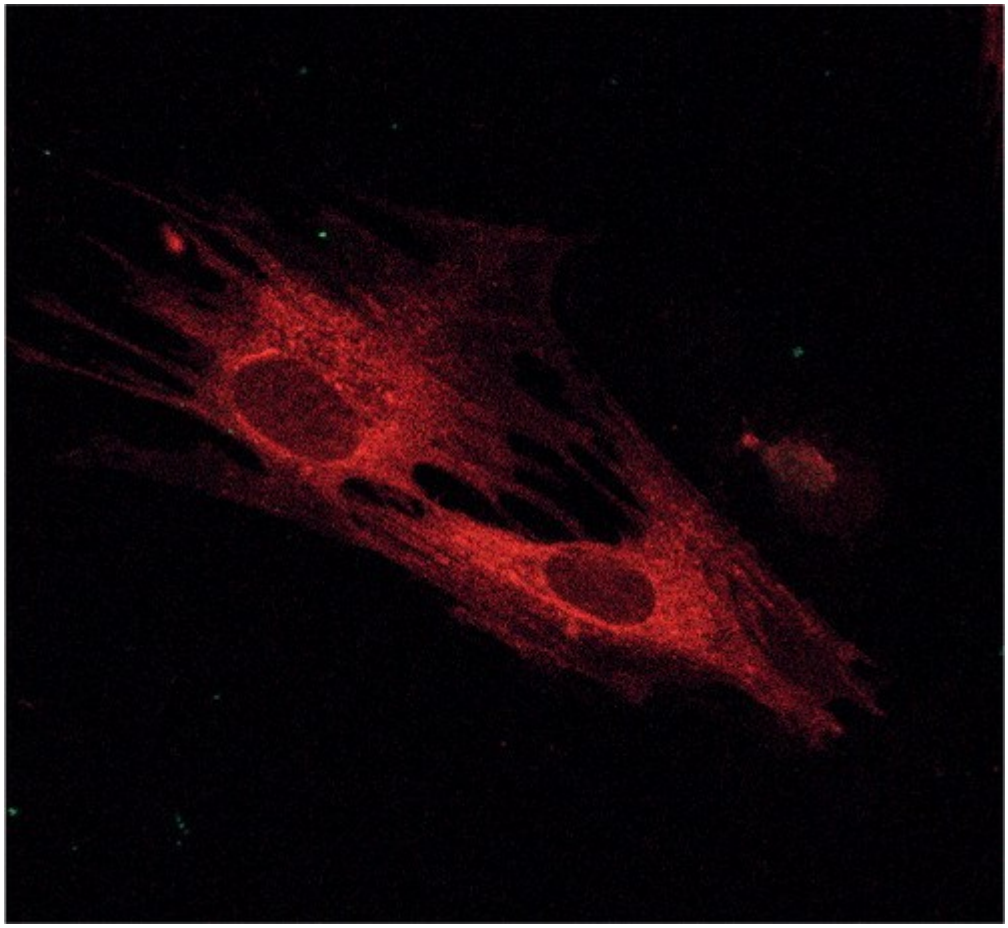


Figure 2 - Flow diagram of the sequence of events for the treatment of tendinopathy using autologous mesenchymal stem cells derived from bone marrow.

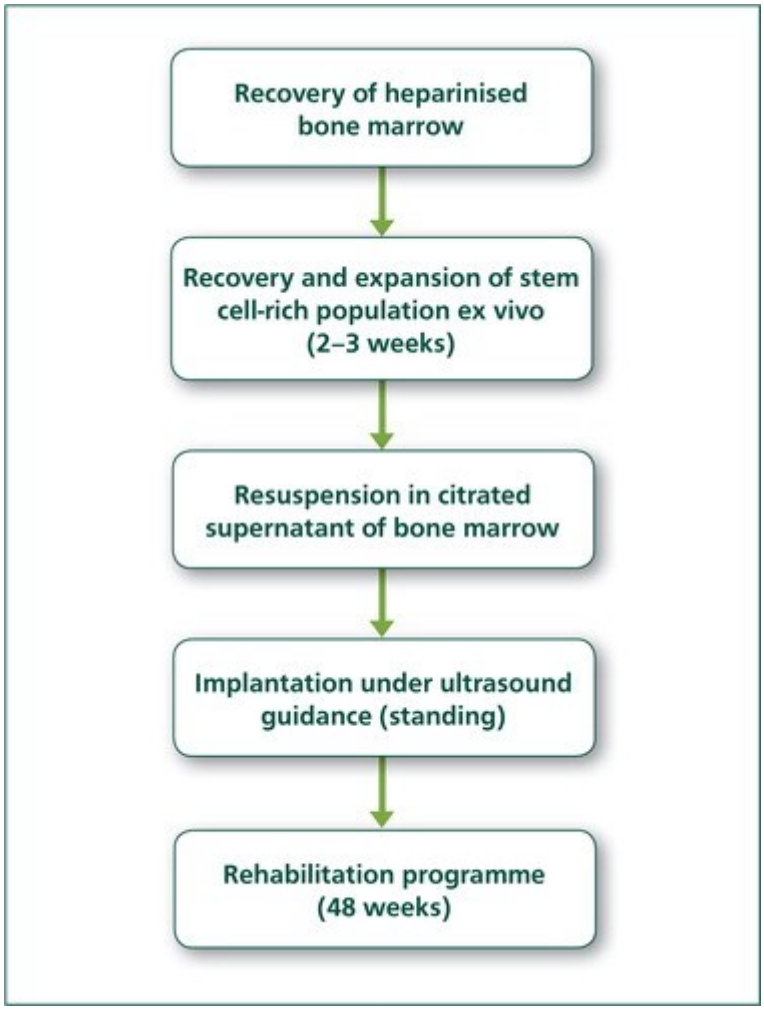
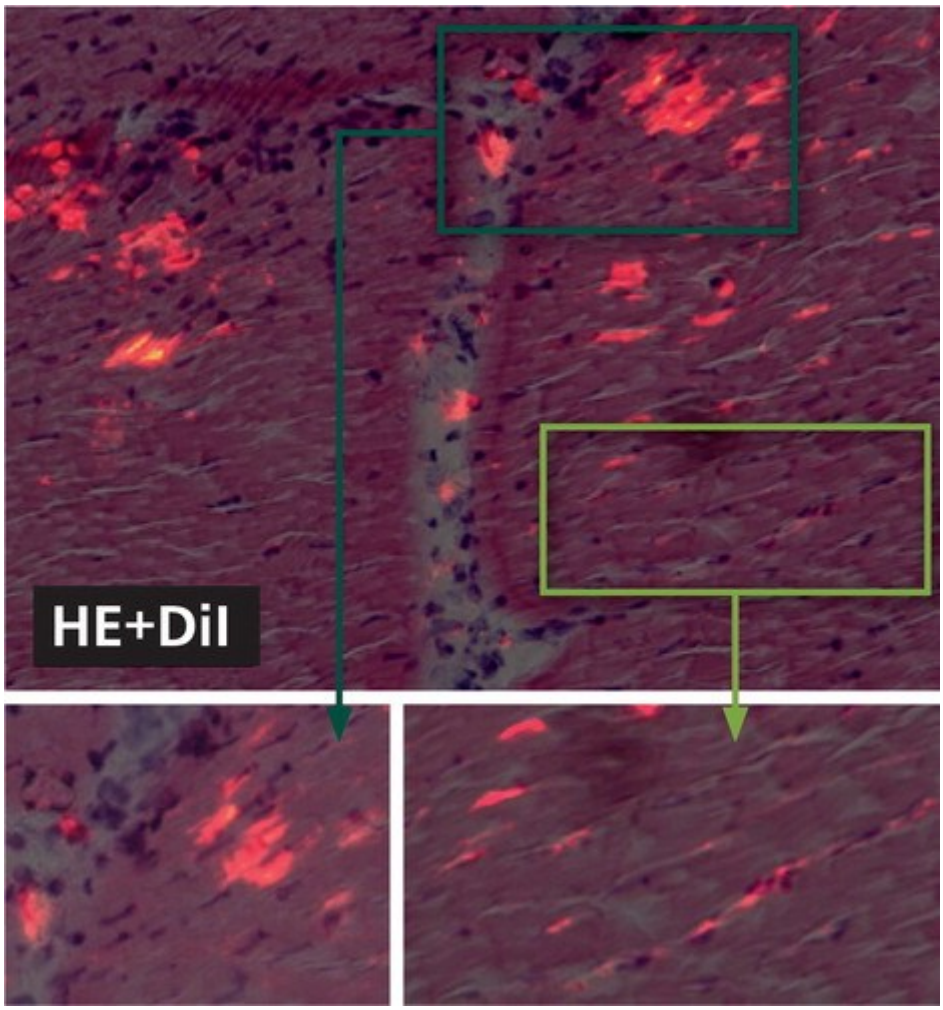


Figure 3 – The distribution of autologous equine mesenchymal stem cells, labelled with Di-I, after implantation into a naturally-occurring superficial digital flexor tendinopathy. Note that the number of stem cells retained is not high and they are distributed mostly within the endotenon tissue between the fascicles but also in smaller number within the fascicles. While improving retention might improve efficacy, the paracrine activity of relatively small numbers of MSCs can still be sufficient to influence the healing process in a beneficial way. (Image courtesy of Dr. Yoshinori Kasashima, Japan Racing Association).



Key Points

- Regenerative medicine offers a unique strategy for the treatment o musculoskeletal disease
- 'Regenerative medicine' techniques currently available are rarely, if ever, truly regenerative but have still been shown to improve healing. Hence 'biological therapies' is probably a more accurate term at present.
- The most commonly used biological therapies, prepared from the patient's own blood or tissues, are platelet-rich plasma (PRP), interleukin-1 receptor antagonist protein (IRAP), and mesenchymal stem cells (MSCs)
- Platelet-rich plasma acts by delivering anabolic growth factors
- IRAP acts by delivering growth factors and possibly inhibiting interleukin-1
- Mesenchymal stem cells most likely act through paracrine factors they secrete which are immunomodulatory and influence the inflammatory process to achieve more functional healing
- In equine orthopaedics they are most commonly used for the treatment of tendon and ligament injuries and joint disease

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