Distribution of Injected Technetium^{99m}-Labeled Mesenchymal Stem Cells in Horses with Naturally Occurring Tendinopathy

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ABSTRACT: This study aimed to investigate immediate cell survival and distribution following different administration routes of mesenchymal stem cells (MSCs) into naturally occurring tendon injuries. Ten million MSCs, labeled with technetium-99m hexamethylpropyleneamine oxime, were implanted into 13 horses with naturally occurring tendon or ligament injuries intra-lesionally, intravenously and by regional perfusion, and traced for up to 48 h using planar gamma scintigraphy. Labeling efficiencies varied between 1.8% and 18.5% (mean 9.3%). Cells were retained in the damaged area after intra-lesional administration but only 24% of cells were still present within the tendon after 24 h. After intravenous injection, cells largely distributed to the lung fields, with no detectable cells in the tendon lesions. Significant labeling of the tendon lesions was observed in 11/12 horses following regional perfusion but at a lower level to intra-lesional injection. The highest cell numbers were retained after intra-lesional injection, although with considerable cell loss, while regional perfusion may be a viable alternative for MSC delivery. Cells did not "home" to damaged tendon in large numbers after intravenous administration. Cells were detected in the lungs most frequently after intravascular administration, although with no adverse effects. Low cell retention has important implications for designing effective clinical therapies for human clinical use. 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop

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Mesenchymal stem cells (MSCs), recovered from a variety of tissue sources, have attracted considerable interest in recent years as a potential therapeutic tool to achieve regeneration after injury.[1] This has been supported by a number of experimental studies on the implantation of MSCs into tendons after a variety of experimentally created tendon lesions in laboratory animals which have induced significant beneficial effects on tendon healing.[2–6]

Because the horse is used for many forms of athletic endeavor, it suffers a high frequency of age-related over-extension injury to the supporting tendons of the distal forelimb, in particular the superficial digital flexor tendon (SDFT) which acts as an elastic energy store, similar to the Achilles tendon in humans.[7] While these injuries heal with fibrosis, the resulting tendon is less functional than the original[8,9] with a resulting high frequency of re-injury.[10,11] Thus, with the potential of influencing natural repair using MSCs based on experimental data, a technique was developed to treat naturally occurring tendinopathy in horses using autologous MSCs.[12] The technique has grown in popularity[13] with recent evidence of efficacy supported by both experimental[14,15] and clinical studies.[16]

The survival of intra-lesionally implanted MSCs has been investigated using labeled cells and subsequent evaluation of the tissue up to 3–4 months after implantation has demonstrated persistence of implanted cells but only in relatively small numbers.[17,18] However, it is not clear when this loss occurs and how many survive the initial implantation process. Furthermore, alternative "remote" injection routes have been suggested because of the potential "homing" ability of MSCs.

Iron oxide nanoparticles have been used to follow injected cells in vivo using MRI as have radionuclides.[19,20] Of the radionuclides, both indium^{111} and technetium^{99m} (Tc^{99m}) have been used.[20] Indium labels cells non-specifically and no additional linker is required. However, cell viability is lower with indium[21] and, due to its longer half-life, has radiation protection safety issues in equine practice. In contrast, Tc^{99m} is widely used in horses for gamma scintigraphy and has also been used to label cells ex vivo for the identification of septic foci by labeling white blood cells[22,23] using hexamethyl-propyleneamine oxime (HMPAO) as a linker molecule to bind the technetium, as Tc^{99m}-HMPAO, to cells and for the monitoring of stem cell fate in horses.[24]

For this study, we elected to use Tc^{99m}-HMPAO to label MSCs ex vivo prior to injection and to follow their distribution in horses with naturally occurring tendinopathy. The measurement of the radioactivity would determine the persistence of injected MSCs over a 24 h period. We hypothesized that MSCs would be retained within the tendon after intra-lesional injection and that MSCs can "home" to the sites of injury in digital flexor tendons when injected remotely either by intravenous injection into the jugular vein or via the digital veins while a proximal tourniquet is in place (regional perfusion).

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Table 1. Case Details of the Horses Used in This Study

Horse Number	Breed	Age (Years)	Sex	Injury Site and Extent of Lesion (cm DACB)	Duration to IV (Weeks)	Duration to RP (Weeks)	Duration to IL (Weeks)
1	Andalusian	10	F	Left SDFT 3–10	6	7	5
2	Polo Pony	12	F	Left SDFT 12–24	4	5	6
3	Polo Pony	8	M	Left SDFT 18–22	7	5	6
4	Selle Francais	19	M	Right SDFT 10–20	8	7	6
5	Polo Pony	9	M	Left SDFT 12–17	6	7	5
6	Unknown cross	5	F	Left SDFT 17–22	8	7	6
7	Andalusian	6	M	Right SDFT 12–18	7	8	9
8	Polo Pony	7	F	Left ALDDFT	9	10	8
9	Polo Pony	12	F	Right SDFT 15–25	9	7	8
10	Polo Pony	9	F	Left SDFT 20–24	10	8	9
11	Polo Pony	13	F	Left ALDDFT	7	8	9
12	Polo Pony	9	F	Left ALDDFT	9	10	8
13	Cross	9	F	Left SDFT 8–14	7	5	6

DACB, distal to accessory carpal bone; IV, intravenous; RP, regional perfusion; IL, intralesional; SDFT, superficial digital flexor tendon; ALDDFT, accessory ligament of the deep digital flexor tendon.

MATERIALS AND METHODS

Optimizing Labeling Efficiency

MSCs were isolated and prepared from two horses as described (see below (16, 24)) and used at passage 2 for labeling. One thousand MBq of Tc^{99m} pertechnetate (Tc^{99m}) in 0.5–0.7 ml was incubated with 0.5 mg (1 vial) of hexamethylpropyleneamine oxime (HMPAO; Ceretec[1]) for 10 min. After this time, 300–500 MBq Tc^{99m}-HMPAO was added to 10 million MSCs either undiluted or in 1 ml of either phosphate buffered saline (PBS, without Ca^{+} or Mg^{+}; from PAA, U.K.) or Dulbecco's Modified Eagles Medium (DMEM, high glucose; from PAA, U.K.[2]) and incubated at room temperature for 10, 20, or 30 min. After these incubation times, the cells and Tc^{99m}-HMPAO mixture was centrifuged at 400*g* for 5 min and the cells washed with 10 ml of fresh DMEM before counting the radioactivity in both supernatants and the cells separately. From these values, the labeling efficiency (percentage of starting radionuclide bound by the cells) was calculated using the standard formula for cell labeling with Tc^{99m}-HMPAO[20]: (radioactivity of cells)/(radioactivity of cells + supernatant) × 100. As the total radionuclide (Tc^{99m}-HMPAO molecules) was in an excess amount of the cell population, it was assumed that all the cells were labeled and that the distribution of label was even per cell. Post-labeling cell viability was determined using the trypan blue (Sigma–Aldrich) exclusion test within 30 min.

Cases

Thirteen horses were utilized in this study. These horses were all presented with a non-traumatic over-strain injury (tendinopathy or desmopathy) to one of the palmar soft tissue supporting structures of the metacarpal region of the forelimb (12 of 13 horses) or metatarsal region of the hind limb (1 of 13 horses; Table 1). Both the affected and contralateral limbs were palpated and examined ultrasonographically to confirm the diagnosis. For inclusion in the study, lesions had to have occurred less than 21 days prior to presentation. This, together with a small variation in the time for cell expansion in culture, is reflected in the different intervals to first injection for each horse noted in Table 1. Ethical permission for this study was granted by the Animal Ethics and Welfare Committee of the Colegio de Veterinarios de Malaga, Spain.

Recovery of MSCs

Two 9.5 ml aliquots of bone marrow (in 250 iu/ml heparin) were harvested from the sternum in the standing horse under sedation and following a standardized protocol.[16,25] Autologous MSCs were isolated and expanded using the protocol described previously.[12] In brief, MSCs were recovered by adherence to tissue culture plastic and expanded in DMEM containing 10% fetal calf serum (FCS; from PAA, U.K.). Cells were passaged once over approximately a 3 week period before three separate 10 million cell aliquots were suspended in autologous citrated bone marrow supernatant, for transport to the clinic on three consecutive weeks, for labeling and implantation.

Labeling of MSCs for Clinical Study

The cell suspensions were first centrifuged for 5 min at 400*g*. The bone marrow supernatant was recovered and transferred to a new and sterile microcentrifuge tube which was kept chilled. The cell pellet was resuspended in the residual supernatant.

The Tc^{99m}-HMPAO was prepared separately by adding 1–2 ml of Tc^{99m} (1,000 MBq) to 1 vial of HMPAO, which was incubated in a lead-lined radio-isotope container for 5–10 min. This Tc^{99m}-HMPAO mixture was added to the pelleted MSCs and mixed by gentle shaking. This preparation was then incubated for 30 min at room temperature after which the preparation was spun for 5 min at 400*g*. The supernatant was recovered and the cells were washed with 1 ml of PBS, spun and separated identically. The radioactivity was recorded for both supernatants and the final cell pellet and the labeling efficiency calculated from the formula:

$$\frac{\text{Radioactivity of cell pellet}}{\text{Radioactivity of supernatant 1} + \text{supernatant 2} + \text{cell pellet}} \times 100$$

The cell pellet was then resuspended in 1 ml PBS. When the MSCs were implanted intralesionally the cell pellet was

[1]GE Healthcare, Hatfield, UK.
[2]Sigma–Aldrich, UK.

resuspended in 1 ml of the original bone marrow supernatant and injected into the horse according to the protocol described below.

Implantation Protocol

Ten million autologous MSCs labeled with Tc^{99m}-HMPAO were administered to each horse by each of three randomized routes every 7 days as follows:

1. Intralesionally under ultrasonographic guidance using a 1.5 in. 20 gauge needle at one site at the maximum injury zone. (intralesional—IL; $n = 13$).
2. Intravenously by first diluting the MSCs in 19 ml PBS and administering it via the lateral palmar digital vein in the pastern region while a rubber tourniquet was applied in the proximal metacarpal region (regional perfusion—RP; $n = 12$). The tourniquet was left in place for 30 min after injection before release.
3. Intravenously via a preplaced catheter in the jugular vein (intravenous—IV; $n = 13$).

The activity of Tc^{99m} used for labeling for each of the administration routes used were 1,000, 2,000, and 7,000 MBq for intralesional, regional perfusion and intravenous, respectively. The rationale for the order of cell injection (IV-RP-IL vs. IL-IV-RP) was influenced by the local regulations that limited the amount of radiation received per animal each day (10 GBq/day of Tc^{99m}), making it necessary to arrange a randomized combination of IL-RP-IV (1, 2, and 7 GBq/day respectively). Injection of labeled MSCs was chosen to be at 7 days to ensure no ionizing label remained at the time the next injection route was performed and also at least 1 week was necessary to culture expand a further 10 million autologous MSCs for administration.

Imaging Protocol

Planar gamma scintigrams using a gamma camera[3] were obtained within 5 min of injection (IL and IV) or tourniquet release (RP; time 0) and then at 1, 3, 6, 12, 24, 36, and 48 h after administration of Tc^{99m}-HMPAO labeled MSCs. Images were obtained from the lesion area, the equivalent area of the contralateral limb, the left lung field, and the left thyroid. Images were displayed and analyzed using software[4]. Radioactivity levels at the lesion site were measured by analyzing identically-sized regions of interest for each animal at the different time-points, which were then expressed as a percentage of the ROI at time 0, having corrected for the predicted decay of the Tc^{99m}, to give the percentage of cells remaining.

RESULTS

Cell labeling efficiency in a laboratory setting varied from 9% to 55%. Maximum cell labeling of 55% was achieved with 30 min incubation in a small volume (<0.5 ml) of DMEM ($n = 2$). Post labeling cell viability was 92% ($n = 2$) and the ability of the cells to proliferate in in vitro culture remained unaffected (data not shown). In contrast, cell labeling efficiencies in the clinic varied between 1.3% and 18.5% with a mean of $7.2 \pm 3.6\%$ for all cell labeling assays ($n = 39$). Specifically, the mean labeling efficiency was $9.3 \pm 3.7\%$ for intralesional administration, $7.5 \pm 2.5\%$ for regional perfusion and $4.0 \pm 2.3\%$ for intravenous administration.

Distribution of Cells After Implantation

Intralesional Administration

Diffuse uptake of radiopharmaceutical was imaged immediately after implantation throughout the lung field in one horse, in a single focal area within the lung field in two other horses and in multiple focal areas in one horse. In the remaining nine horses, no uptake of radiopharmaceutical was evident within the lungs. No uptake of Tc^{99m} was evident in the thyroid (as free pertechnetate) or within the contralateral limb. Focal uptake was evident at the site of the injection in all 13 horses (Fig. 1). No proximodistal spread was evident after masking the injection site in 6/13 horses; 4/10 horses with SDFT lesions and 2/3 horses with ALDDFT lesions. In one horse (horse 11; ALDDFT lesion), the spread was diffuse proximally. In the other SDFT lesions, 4/13 horses had diffuse distal spread, 1/13 had proximodistal spread, and 1/13 had a focal proximal spread separated from the injection site.

Regional Perfusion

Diffuse uptake throughout all lung fields was identified in 8/12 (67%) horses (Fig. 2). In four horses, foci of radiopharmaceutical uptake were identified. Radiopharmaceutical uptake in the thyroid region was mild in all horses apart from one horse in which the uptake was marked. In the contralateral limb, no uptake of Tc^{99m}-HMPAO was evident in any horse. Radiopharmaceutical uptake was identified at the site of the lesion in 9/12 horses (75%), consisting of 7/9 (78%) of SDFT lesions and 2/3 (66%) of ALDDFT lesions (Fig. 1). All three horses with no uptake at the lesion site had one or two intense focal areas of uptake in other regions of the limb and in the lungs.

Intravenous Administration

Marked diffuse distribution throughout all lung fields was evident at 5 min in 10/13 (77%) of horses; a milder diffuse distribution was seen in the remaining three horses. In all but one horse, the gamma camera could not detect Tc^{99m} within the lung fields after 24 h. In the contralateral limb, no uptake of Tc^{99m} was evident in any horse. Only one horse (horse 3) showed an area of focal uptake in the region of its SDFT lesion which was evident within 5 min of administration of the labeled cells and persisted for 24 h.

Quantification of Cell Persistence After Intralesional Administration

When taking into account the normal decay of Tc^{99m}, there was a rapid drop off in the percentage of cells remaining at the lesion site after both intralesional

[3]GE-400A, GE Healthcare, Hatfield, UK.

[4]MicasX plus, Bartec Medical Imaging Solutions, Surrey, UK.

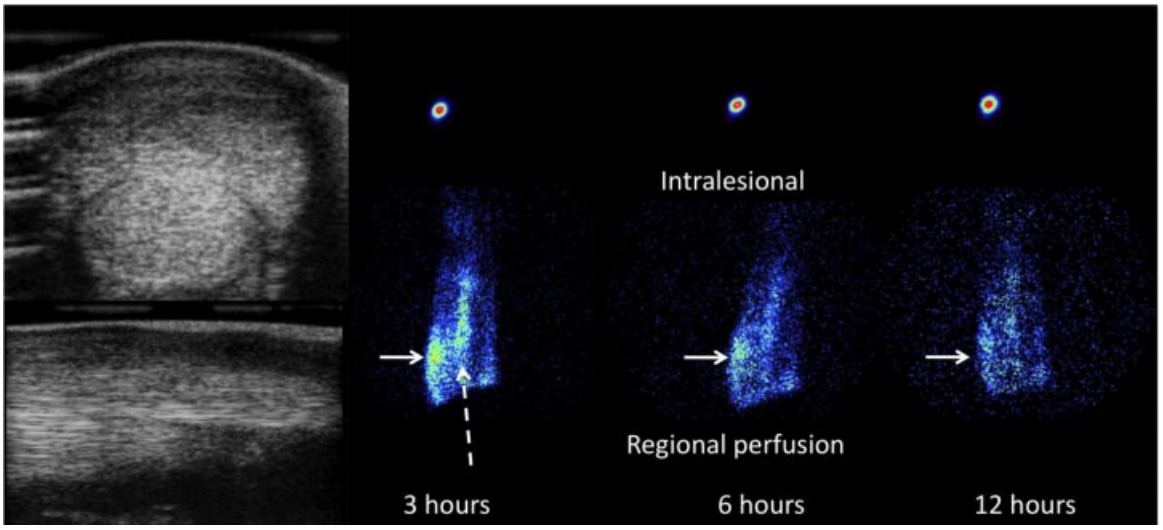

Figure 1. Gamma scintigraphs from a lesion on the distal superficial digital flexor tendon 22 cm distal to the accessory carpal bone (representative ultrasonographs shown on the left). They were performed at 3, 6, and 12 h after administration intra-lesionally (top row) and by intravenous regional perfusion (via the palmar digital vein in the pastern region; bottom row). Note the uptake of radiopharmaceutical over the distal superficial digital flexor tendon at the lesion site (solid arrow). The uptake denoted by the dashed arrow is persistence of uptake in the palmar digital vein.

and regional perfusion (Fig. 3), with an average of 32% and 24% retained at 12 and 24 h, respectively, after intralesional administration compared to 9% retained after 12 h with regional perfusion (Fig. 4).

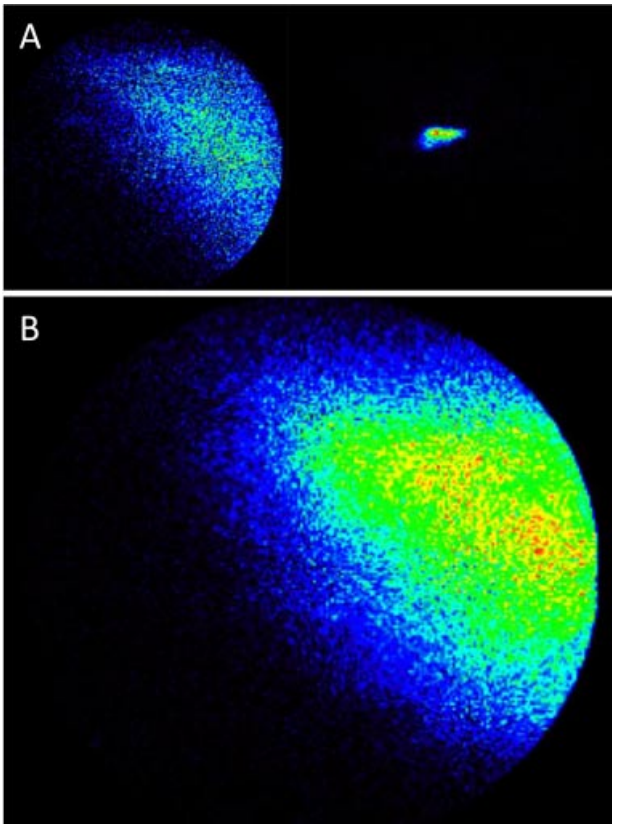

Figure 2. Cell distribution in the lungs after regional perfusion (A) and intravenous administration (B). Note the generalized (A, left) and focal (A, right) distribution seen only occasionally after regional perfusion while all horses showed generalized distribution after intravenous administration.

DISCUSSION

There were substantially lower labeling efficiencies during the clinical phase of the study compared to that achieved during the in vitro phase of the project and reported for the labeling of white blood cells in horses[26,27] and in the labeling of human MSCs with Tc^{99m}.[28] There are two likely explanations for this lower labeling efficiency. Firstly, in the authors experience cells labeled immediately following preparation from blood or organ samples take up label more efficiently than cells that have been previously cul-

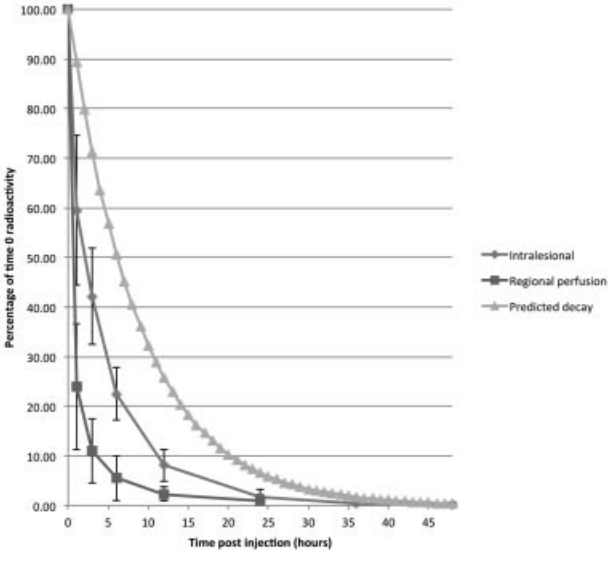

Figure 3. Decline of radioactivity recorded from regions of interest over the lesion in 13 horses when cells were administered either by intralesional injection or regional perfusion. A comparison with the natural decay in radioactivity of Tc^{99m} is shown.

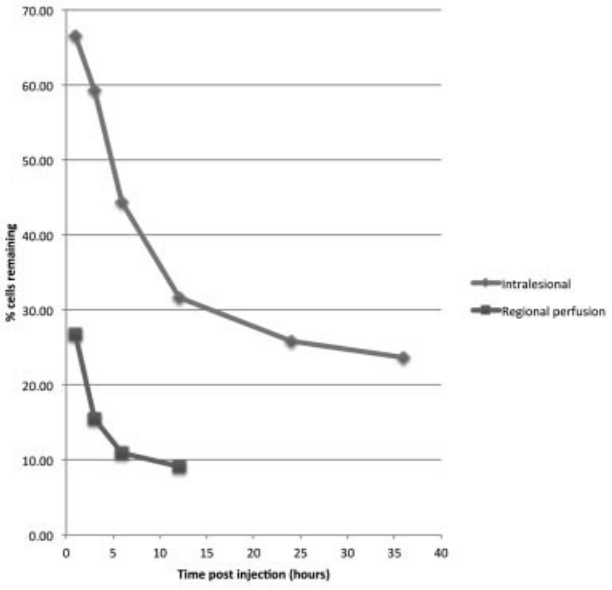

Figure 4. Percentage of cells remaining, as estimated from radioactivity levels in identical regions of interest, after intralesional injection and regional perfusion.

tured. The second, and the most likely reason, may be related to the longer transport time of the Tc^{99m} to the clinic from the supplier and therefore a longer period between elution and use of the Tc^{99m} in the clinical study. Consequently, there would have been appreciable decay of the Tc^{99m} and as the radiochemical purity of Tc^{99m} is reduced after 2 h post-elution (supplier's data sheet) this would result in interference with the binding process of the HMPAO. In similar studies in normal horses[24] labeling efficiencies were higher because these studies were performed in a laboratory environment. However, in the current study, there was sufficient binding to be able to identify cells after implantation for both intra-lesional and regional perfusion administration routes and hence represented adequate labeling efficiency. The absence of any detectable signal at the site of the lesion after intravenous administration in 12/13 horses may, however, be related either to this poor labeling efficiency, insufficient cells administered via this route, or else be due to the absence of significant "homing" of stem cells. It is unlikely that cell migration was influenced by Tc^{99m}-HMPAO labeling as studies in rats with brain or heart lesions have demonstrated that cell migration was independent of labeling by this moiety.[29,30] In further support, the retention levels calculated in this study coincide well with the percentage cell retention calculated from cytoplasmic membrane dyes (Kasashima et al., unpublished observations) and genetic labels[17] at longer duration time-points in horses, suggesting that the labeling method does not adversely influence cell migration itself. Furthermore, Tc^{99m}-HMPAO is a neutral lipophilic chelate which is internalized into the cytoplasm by passive diffusion, where it is modified into a hydrophilic moiety and hence trapped inside the cell. Consequently, it is unlikely to influence cell adhesion since the label is internal and not bound to the cell surface.

The distribution of cells to the lungs after intravenous route was not surprising given that that has been observed after white blood cell labeling.[22,27] This was also observed after intravenous stem cell administration in rats,[28] although subsequent redistribution was observed from the lungs between 2 and 20 h after administration. This remains a possibility in the horse after 24 h but was not observed within the detectable time limit of the Tc^{99m}. There was occasional focal uptake in the lungs after regional perfusion which is most likely to reflect clumping of cells within the venous system which are then released after the tourniquet is removed and are "filtered out" by the lung microvasculature. However, the presence of cells in the lungs was not associated with any adverse clinical signs and all horses were successfully rehabilitated back to performance, an important finding with regards to the safety of these administration routes for humans. The current study was designed to randomize the order of the delivery routes to avoid any consistent influence of one delivery route. In addition, in view of the low retention levels it is unlikely that the number of cells retained after 7 days would influence the retention of cells injected via a different route. The cell retention calculated after intra-lesional injection when intra-lesional injection was carried out first ($n = 8$) and compared that with those horses where regional perfusion was carried out before intra-lesional injection ($n = 5$) showed that there was no significant difference (44.95% vs. 43.70% at 6 h). Given that IV injection resulted in no significant presence of label at the lesion site this supports our view that this will not have influenced the cell retention from more local delivery routes.

This technique estimated that only approximately one quarter of the cells were still present after 24 h after intra-lesional implantation, which is identical to that reported for experimental lesions (personal communication, Dr. M.A. Vidal, University of California-Davis, CA). This figure may represent a low estimate as it is not possible to rule out significant dissociation of the label from the cells when in vivo, although in a previous study, label persistence was evaluated in equine MSCs in vitro and was found to be 83% after 6 h.[24] Tc^{99m}-HMPAO dissociated from the cells is shed via the gut and kidney while free Tc^{99m} is taken up preferentially by the thyroid (supplier's data sheet), which was the reason for evaluating the thyroid at each examination. However, consistent with relatively low levels of label dissociation, there was relatively infrequent identification of free Tc^{99m} in the thyroid. This also suggested that there was no significant effect of labeling on cell viability after implantation as label shed from dead cells would accumulate in the thyroid.

This was consistent with the high cell viability after labeling and the ability of cells to survive and proliferate in our in vitro culture assays. While we cannot determine the effect on the therapeutic efficacy of the cells post-implantation, our previous work has indicated that the MSCs implanted into tendon lesions remain therapeutically effective based on the improved outcome measures.[16]

This level of cell retention, however, is consistent with other labeling studies which estimated less than 5% of the implanted cells still present 10 days after implantation.[18] No dose response exists for the administration of MSCs for the treatment of naturally occurring tendinopathy and so it is not known how many cells are required to have an effect. Although a large percentage of cells appear to be lost soon after implantation, only smaller numbers may be necessary to exert paracrine influences on the resident cells.

Regional perfusion appeared to deliver significantly lower cell numbers than intralesional administration. Higher doses may therefore be necessary to improve cell retention via this route, although it is not possible from this study to determine whether the administered cells were able to exit the blood vessels into the tissue itself, which would seem to be a prerequisite for them to have an effect. Failure of radiopharmaceutical uptake in the lesion in 3/12 cases was accompanied by multiple intense focal uptake over the limbs and in the lungs, probably corresponding to large intravascular clot formation containing labeled cells during the regional perfusion procedure. The effects of injury site and duration of injury on the outcomes are likely to be minimal due to the restricted period of recent onset of the injuries and the restriction of injuries used to the superficial digital flexor tendon and accessory ligament of the deep digital flexor tendon—two similar structures in the same region of the limb. The effect of side of injury (left vs. right) is unlikely to have any effect as affected limb was evenly matched and is not associated with different outcome in tendon injuries in quadrupeds such as horses. Only 1 out of the 13 horses had a hind limb injury and no peculiarities of cell distribution with any of the injection routes were detected in this case.

This article serves as a useful comparison with the data obtained for intravenous and regional perfusion in both normal horses[24] and after experimentally created surgical lesions (personal communication, Dr. M.A. Vidal). While labeling efficiencies were higher in these studies, the conclusions were similar in both studies.

The greatest retention of cells was after intralesional administration compared to regional perfusion, indicating that this is still the most effective route for administering cells, although if no central lesion exists for intra-lesional administration, regional perfusion may be a viable, although less effective, delivery alternative. The relatively low retention of cells has important implications for designing effective clinical therapies and techniques to retain more cells after implantation would be highly relevant to developing this technology for human clinical use.

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