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

Keywords: tendon; stem cell; tendinopathy; cell labeling; technetium

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. 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.

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. While these injuries heal with fibrosis, the resulting tendon is less functional than the original with a resulting high frequency of re-injury. 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. The technique has grown in popularity with recent evidence of efficacy supported by both experimental and clinical studies.

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. 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. Of the radionuclides, both indium (111) and technetium (99m) have been used. Indium labels cells non-specifically and no additional linker is required. However, cell viability is lower with indium 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 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.

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).
MATERIALS AND METHODS

Optimizing Labelling Efficiency

MSCs were isolated and prepared from two horses as described (see below (16, 24)) and used at passage 2 for labelling. One thousand MBq of Tc99m pertechnetate (Tc99m) in 0.5–0.7 ml was incubated with 0.5 mg (1 vial) of hexamethylpropyleneamine oxime (HMPAO; Ceretec1) for 10 min. After this time, 300–500 MBq Tc99m-HMPAO was added to 10 million MSCs either undiluted or in 1 ml of either phosphate buffered saline (PBS, without Ca2+ or Mg2+; from PAA, U.K.) or Dulbecco’s Modified Eagles Medium (DMEM, high glucose; from PAA, U.K.) and incubated at room temperature for 10, 20, or 30 min. After these incubation times, the cells and Tc99m-HMPAO mixture was centrifuged at 400 g for 10 min. This Tc99m-HMPAO mixture was added to the ALDDFT, accessory ligament of the deep digital flexor tendon.

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

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

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.

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; Table 1). Both the affected and contralateral limbs were palpatated 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 400g. 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 Tc99m-HMPAO was prepared separately by adding 1–2 ml of Tc99m (1,000 MBq) to 1 vial of HMPAO, which was incubated in a lead-lined radio-isotope container for 5–10 min. This Tc99m-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 400g. 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 was calculated from the formula:

Radioactivity of cell pellet
Radioactivity of supernatant 1 + supernatant 2 + cell pellet
× 100

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

1GE Healthcare, Hatfield, UK.
2Sigma–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 eastern 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 intrave-
nous, 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 soft-
ware\(^4\). 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 ± 3.6% for all cell labeling assays
(\( n = 39 \)). Specifically, the mean labeling efficiency
was 9.3 ± 3.7% for intraleSIONal administration,
7.5 ± 2.5% for regional perfusion and 4.0 ± 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 identi-
fied in 8/12 (67%) horses (Fig. 2). In four horses, foci of
radiopharmaceutical uptake were identified. Radiophar-
maceutical 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. Radiophar-
maceutical 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.
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).

**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 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.

**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.

**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.
Migration itself. Furthermore, Tc99m-HMPAO is a labeling method does not adversely influence cell duration time-points in horses, suggesting that the studies in normal horses labeling efficiencies were with the binding process of the HMPAO. In similar (supplier’s data sheet) this would result in interference between elution and use of the Tc99m in the clinical clinic from the supplier and therefore a longer period was influenced by Tc99m-HMPAO labeling as “homing” of stem cells. It is unlikely that cell migration or else be due to the absence of significant efficiency, insufficient cells administered via this may, however, be related either to this poor labeling lesion after intravenous administration in 12/13 horses absence of any detectable signal at the site of the hence represented adequate labeling efficiency. The and regional perfusion administration routes and identify cells after implantation for both intra-lesional study, there was sufficient binding to be able to filtration in rats,28 although subsequent redistribution retention calculated after intra-lesional injection when 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. Tc99m-HMPAO dissociated from the cells is shed via the gut and kidney while free Tc99m 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 Tc99m 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.16 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 intraleseional 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 peculiarity 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 horses24 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 intralesional 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.

ACKNOWLEDGMENTS
R.K.W.S. was a technical adviser for VetCell. The authors would like to thank the referring veterinarians and owners for referring horses for treatment in this study and the logistic efforts displayed by Molypharma Spain.

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