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Title

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Permalink

https://escholarship.org/uc/item/2tk5q3jg

Journal

Cell and tissue research, 340(1)

ISSN

1432-0878

Authors

Genetos, Damian C
Rao, Rameshwar R
Vidal, Martin A

Publication Date

2010-04-18

Peer reviewed

Scintigraphic comparison of intra-arterial injection and distal intravenous regional limb perfusion for administration of mesenchymal stem cells to the equine foot

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Summary

Reasons for performing study: Intra-arterial (i.a.) and intravenous (i.v.) regional limb perfusions (RLP) through the median artery and cephalic vein, respectively, have been previously investigated for administration of mesenchymal stem cells (MSCs) to the equine distal limb. Limitations due to thrombosis of the arteries after i.a. RLP and poor distribution of MSCs to the foot with i.v. RLP were observed. These techniques need to be modified for clinical use.
Objectives: Evaluate the distribution, uptake and persistence of radiolabelled MSCs after i.a. injection through the median artery without a tourniquet and after i.v. RLP through the lateral palmar digital vein.
Study design: *In vivo* experimental study.
Methods: ^{99m}Tc-HMPAO-labelled MSCs were injected through the median artery of one limb and the lateral palmar digital vein of the other limb of 6 horses under general anaesthesia. No tourniquet was used for the i.a. injection. A pneumatic tourniquet was placed on the metacarpus for i.v. injection. Scintigraphic images were obtained up to 24 h after injection.
Results: Intra-arterial injection resulted in MSCs retention within the limb despite the absence of a tourniquet and no thrombosis was observed. Both i.a. injection and i.v. RLP led to distribution of MSCs to the foot. The i.a. injection resulted in a more homogeneous distribution. The MSC uptake was higher with i.v. RLP at the initial timepoints, but no significant difference was present at 24 h.
Conclusions: Both i.a. injection through the median artery without a tourniquet and i.v. RLP performed through the lateral palmar digital vein under general anaesthesia are safe and reliable methods for administration of MSCs to the equine foot. The i.a. technique is preferred owing to the better distribution, but is technically more challenging. The feasibility of performing these techniques on standing horses remains to be investigated.

Keywords: horse; mesenchymal stem cells; regional limb perfusion; scintigraphy; hexamethylpropyleneamine oxime; HMPAO; technetium

Introduction

Mesenchymal stem cell (MSC) therapy for orthopaedic injuries is common in equine clinical practice [1]. Tendon and ligament injuries are the most common orthopaedic disorders treated with cellular therapy [2,3]. The ultimate goal is to improve healing quality and reduce healing time and injury recurrence rate [4,5]. Intralesional administration has been the main route of administration for MSCs into injured tendons and ligaments [2,3,6]. However, this technique has limitations related to a risk of iatrogenic damage [7,8] and the difficulty of targeting lesions in certain areas, such as the hoof capsule [9].

Recently, intravenous (i.v.) and intra-arterial (i.a.) regional limb perfusions (RLP) of MSCs have been proposed as an alternative route for treatment of orthopaedic soft tissue injuries [10–12]. Scintigraphic studies using technetium-HMPAO for labelling of MSCs have demonstrated persistence of MSCs in perfused tissue 24 h after administration [10–12]. Limitations that need to be addressed prior to clinical use of the RLP techniques have been identified. The main limitation of the i.v. RLP technique with injection through the cephalic vein was an inconsistent distribution of MSCs to the foot and pastern area [10]. The i.a. regional limb perfusion performed through the median artery had a more reliable cell distribution in the pastern and foot area, but was associated with a risk of serious complications due to thrombosis of the medial palmar artery [10,11]. These studies suggested that both the distribution of MSCs to the foot after i.v. RLP and the safety of the i.a. RLP needed to be improved before regional perfusion of MSCs could be recommended for clinical use.

We hypothesised that i.v. RLP through the lateral palmar digital vein would improve distribution of the MSCs to the foot and pastern compared to i.v. RLP through the cephalic vein, and that i.a. administration of MSCs without a tourniquet would reduce the risk for thrombosis but still result in persistence of MSCs in the distal limb. The objectives of our study were to assess the distribution, uptake and persistence of MSCs in the foot and pastern area after i.v. RLP through the lateral palmar digital vein and i.a. injection through the median artery using HMPAO labelling and scintigraphy.

Materials and methods

Animals

Six geldings (4 Thoroughbreds, 1 Quarter Horse and 1 Warmblood) with no signs of lameness or musculoskeletal injury were included in the study. Mean age was 13.6 ± 5.4 years (range 6–22 years) and mean bodyweight (bwt) 577 ± 627 kg (range 508–627 kg). The protocol was approved by the Institutional Animal Care and Use committee. Horses were placed under general anaesthesia for MSC administration and initial imaging timepoints. They were sedated with i.v. xylazine (Anased)[a] (1.1 mg/kg bwt) and butorphanol (Torbugesic)[b] (0.1 mg/kg bwt) and general anaesthesia was induced with i.v. ketamine (Ketved)[c] (2 mg/kg bwt) and diazepam[d] (0.05 mg/kg bwt) and maintained using isoflurane[e] in oxygen. Three horses were positioned in left lateral recumbency and 3 in right lateral recumbency. Horses recovered from anaesthesia without assistance after acquisition of the images 60 min after administration.

Mesenchymal stem cell source and culture

Allogeneic bone marrow-derived MSCs were obtained from the sternum of a separate donor. The bone marrow was collected and MSCs isolated and expanded as previously described [13,14]. Cryopreserved MSCs (~1×10^6) of passage 2–4 were thawed and seeded at ~5700 cells/cm² in a T175 flask (Cellstar cell culture flask)[f] and cultured for 3 days. Cells were then passaged after obtaining ~80% confluence, seeded into 10 T175 flasks at ~3000–4000 cells/cm² and expanded for 3–4 days.

Mesenchymal stem cell labelling technique

The MSCs were labelled using a modification of a protocol previously described [10]. Briefly, hexamethylpropyleneamine oxime (HMPAO) was solubilised in 20 µl DMSO and then diluted to 125 µg/ml with sodium chloride injection solution. Approximately 20 mCi (740 MBq) of technetium-99 (^{99m}Tc) and 7.6 µg of tin (II) chloride[g] were added to an aliquot of the HMPAO solution.

MSCs (~70 × 10^{6} cells) suspended in 0.5 ml Dulbecco's Modified Eagle Medium (DMEM) were added to the ^{99m}Tc–HMPAO-tin chloride solution. The reaction was allowed to proceed with gentle rocking for 23 min at room temperature. Mesenchymal stem cells were then centrifuged at ~200 *g* for 10 min, washed once with 5 ml sterile saline and centrifuged again. Radioactivity in both the pellet and the supernatant tubes was recorded. Mesenchymal stem cells were resuspended in 4 ml sterile saline. Radioactivity was measured again immediately prior to injection. The labelling efficiency, defined as the radioactivity in the pellet divided by radioactivity in the pellet and the supernatant, was calculated for each horse.

Intra-arterial injection

A 36 mm long 18 gauge catheter was placed into the median artery of the recumbent limb (i.e. right limb if the horse was in right lateral recumbency) at the level of the distal radius under ultrasound guidance using a linear 3–11 MHz probe (Philips iE33)[h]. Allogeneic ^{99m}Tc-HMPAO-labelled MSCs (~35 × 10^{6} cells) suspended in 2 ml of saline were slowly injected through the i.a. catheter and flushed with 5 ml of saline.

Intravenous regional limb perfusion

The i.v. injection was performed 15 min after the i.a. injection. A 24 mm long 20 gauge i.v. catheter was placed into the lateral palmar digital vein of the upper limb (left limb if the horse was in right lateral recumbency) at the level of the proximal sesamoid bones. A pneumatic tourniquet with a 10.5 cm cuff was placed at the mid to proximal metacarpus immediately prior to injection. Inflation pressure of 450 mmHg was used. ^{99m}Tc-HMPAO-labelled MSCs (~35 × 10^{6} cells) suspended in 10 ml of saline were slowly injected through the i.v. catheter and flushed with 10 ml of saline. The tourniquet was removed 30 min after injection.

Scintigraphic protocol

Planar scintigraphic images of both front limbs from the carpus to the distal extremity were acquired with a gamma camera (IS2 Gamma Camera)[i] set at 140 keV photoelectric peak, 20% symmetrical window and equipped with a low energy, parallel hole collimator. The injected dose of radioactivity was measured using a Well counter (Deluxe Isotope Calibrator II)[j] immediately prior to injection.

Lateral images were acquired during the MSC injection using a dynamic mode with 43 frames of 2 s duration and a 128 × 128 matrix. Lateral and dorsal images were acquired in a static mode, with a single 120 s frame and a 256 × 256 matrix immediately after injections (T0), 30 min after injection (T30) (which included immediately prior and immediately after removal of the tourniquet for i.v. RLP), 60 min (T60), 6 h (T6h) and 24 h (T24h) after injection. The images at T0, T30 and T60 were acquired with the horse under general anaesthesia. The T6h and T24h images were obtained in standing horses sedated with xylazine (0.5 mg/kg bwt i.v.).

Scintigraphic image analysis

The regional distribution of the radiolabelled MSCs was assessed by subjectively evaluating the radioactive signal in the metacarpus, fetlock, pastern and foot areas 1 h after MSC administration. The distribution was considered good if a radioactive signal was present throughout the entire area, partial if present in only part of the area and absent if no signal was detected. The pattern of distribution was also assessed as homogeneous or heterogeneous, depending on whether the signal was distributed evenly or if focal areas presented higher signal intensity.

Objective assessment of uptake for each timepoint was performed by drawing a region of interest (ROI) following the contour of the distal extremity (fetlock, pastern and foot) using the freehand ROI tool of OsiriX (OsiriX v.2.7.5 32-bit)[k]. Measurements were corrected for radioactive

decay, based on the time elapsed between T0 and the time of measurement. Background correction was also applied at T24h owing to the lower signal to noise ratio.

A time–activity curve was obtained using the data from the dynamic acquisition. The height of the peak and the height of the plateau were measured to calculate the relative height of the plateau as the ratio plateau height divided by peak height.

The distal extremity percent uptake (% uptake) was calculated as the lesion ROI corrected activity (t) divided by the injected activity, obtained by measuring the syringe activity pre- and post injection:

$$\% \text{ uptake}(t) = (\text{ROI decay corrected activity}(t)/ \\ (\text{activity syringe pre - activity syringe post})) \times 100.$$

The distal extremity percent persistence (% pers) was calculated as the corrected activity in the ROI at the time of interest divided by the activity in the ROI immediately after injection:

$$\% \text{ pers}(t) = (\text{ROI decay corrected activity}(t)/\text{ROI activity at } T_0) \times 100.$$

Ultrasonographic assessment

The vessels of the distal limb from the site of injection (median artery or lateral palmar digital vein) to the distal extremity were examined ultrasonographically with a variable frequency 6–18 MHz linear transducer[l] 2 days after MSC administration.

Data analysis

Wilcoxon's signed ranks test was used to compare both the uptake and the persistence of MSC at each time point for the 2 different techniques.

Results

Mesenchymal stem cell labelling and administration

The labelling efficiency was 59% (median) and ranged between 47% and 71%. All horses were successfully injected and no clinical complications were observed. Each limb was injected with 30.5 (26.0–51.5) million MSCs (median (range)) with a radioactive dose of 4.5 (2.6–5.7) mCi (166 [96–212] MBq).

Mesenchymal stem cell distribution

Radioactive signal was identified up to 24 h in all distal limbs in all horses with both techniques. The radioactive signal was more homogeneous with i.a. injection. Intravenous RLP led to a more heterogeneous pattern, with higher signal localised at the palmar digital veins and coronary plexus (Fig 1). After i.a. injection, the regional distribution was qualified as good for the fetlock, pastern and foot in 5 of 6 horses (Fig 1). In *Horse 6*, uptake in these regions was graded as partial owing to lower signal on the lateral side. The regional distribution in the metacarpal area after i.a. injection was considered partial owing to poor signal at the proximal and dorsal aspects of the metacarpus in 5 of 6 horses. After i.v. RLP, distribution in the fetlock and pastern was partial in all 6 limbs owing to a lack of radioactive signal on the medial side, whereas strong signal was identified in the area of the lateral palmar digital vein (Fig 1). The regional distribution in the foot after i.v. RLP was partial in 4 limbs, again owing to a lack of radioactive signal on the medial side, and good in the other 2 limbs. There was no signal in the metacarpus of limbs injected with i.v. RLP, which was expected owing to the location of the tourniquet.

Mesenchymal stem cell uptake

The time–activity curves obtained after i.a. administration reveal the presence of a plateau at 58 (44–74) % of the injection peak on all 6 limbs, demonstrating retention of labelled MSCs in the limb despite the absence of a tourniquet (Fig 2). Immediately after injection, the uptake with the i.a. technique (28 [20–30] %) is significantly lower than with i.v. RLP (78 [74–80] %) (P = 0.0277). This difference remains significant until 6 h after MSC administration, but no significant difference in uptake is observed at 24 h (Fig 3).
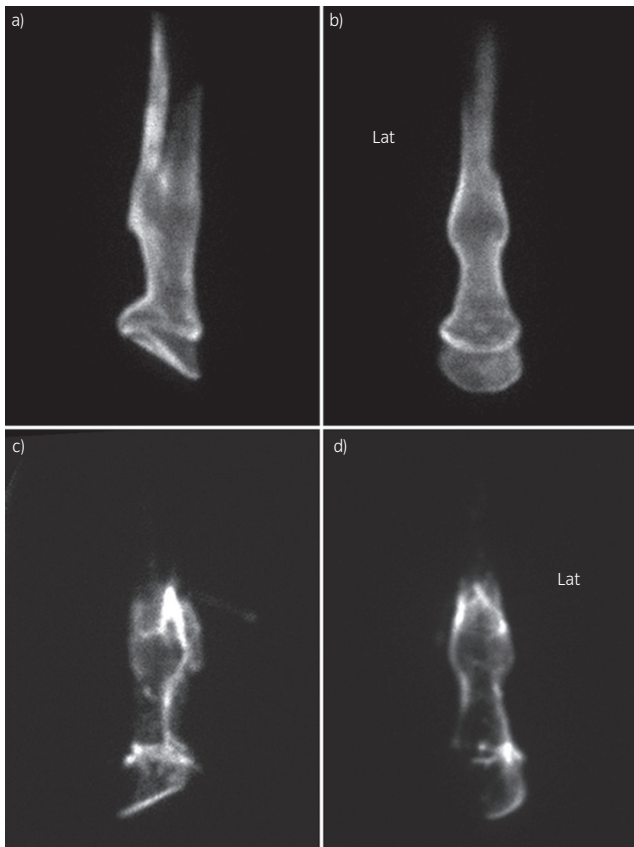
Fig 1: Lateral (a, c) and dorsal (b, d) scintigraphic images of the distal extremity of both front limbs of a horse immediately after i.a. injection of the right limb (a, b) and i.v. RLP of the left limb (c, d). After the i.a. injection (a, b), there is good distribution of the radiolabelled MSCs with radioactive signal present in the entire distal limb. The radioactive signal has a homogeneous pattern of distribution. With the i.v. RLP (c, d), only partial distribution of the radiolabelled MSCs is observed, with absence of radioactive signal at the medial aspect of the distal pastern and foot. The pattern of distribution of the radioactive signal is more heterogeneous, with higher radioactivity in the area of the larger vessels and coronary plexus.

Mesenchymal stem cell persistence

The persistence of the MSCs is reported in Figure 4. After tourniquet removal for i.v. RLP, no significant difference in persistence was observed between the 2 techniques until 6 h after administration, where persistence documented with i.a. injection became significantly higher than with i.v. RLP for both the 6 and 24 h timepoints (P = 0.0277 at 6 and 24 h) (Fig 4).

Ultrasonographic assessment

Ultrasonographic examination of vasculature in both limbs was performed 48 h after injection. No thrombi were identified in the main vasculature of the limbs after i.a. injection. In limbs treated with i.v. RLP, thrombi were identified in arteries of 4 horses. Two showed partial thrombosis of the lateral and medial palmar digital arteries, whereas 2 horses had partial thrombosis of the lateral palmar digital artery only. No clinical signs were associated with these thrombi.

Discussion

This study demonstrated that the previously described techniques of i.a and i.v. RLP of MSCs [10] could both be improved to make them more amenable for clinical use. Performing i.a. injection without a tourniquet resolved the complication of thrombosis observed in previous studies [10,11]. This finding supports that thrombosis was mostly due to blood
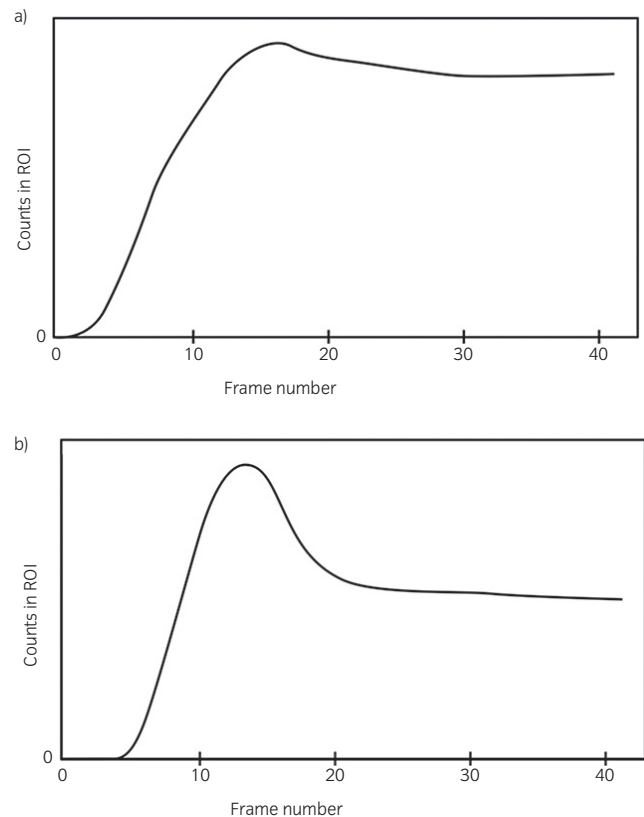

Fig 2: Time–activity curves from a single horse obtained at the time of administration of radiolabelled MSCs using (a) i.v. regional limb perfusion (presence of a tourniquet) and (b) i.a. injection without the use of a tourniquet. With a tourniquet in place (a), the plateau is, as expected, almost at the peak level, demonstrating near complete retention of the MSCs in the limb. With the i.a. injection (b), despite the absence of a tourniquet, a high plateau of radioactivity is observed demonstrating retention of approximately 50% of radiolabelled MSCs in the limb.

stasis induced by the tourniquet and that MSCs only played a secondary role in the thrombogenesis. The limitation of not using a tourniquet was obviously the risk of immediate loss of MSCs to the general circulation. This *in vivo* tracking study demonstrated retention of the MSCs in the distal limb despite the absence of the tourniquet.

This result is in agreement with the observations in rodent models that MSCs tend to be retained by the first capillary bed they encounter;
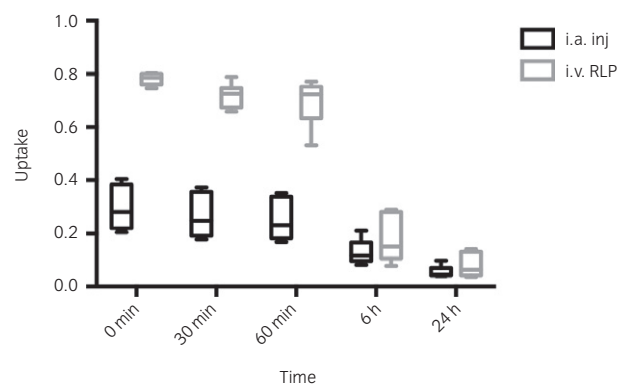

Fig 3: Box-plot representing the radioactive uptake in the distal limb (time corrected activity in the distal limb/injected radioactivity) over time. The uptake is significantly higher with i.v. RLP at the 3 initial timepoints; however, no significant difference is observed at 6 and 24 h after injection.
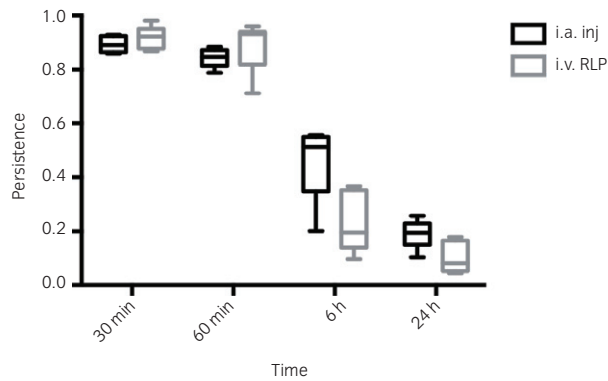
Fig 4: Box-plot representing the radioactive persistence in the distal limb (time corrected activity in the distal limb/initial activity in the distal limb) over time. No significant differences are observed at 30 and 60 min after injection; however, the persistence is higher with i.a. injection at 6 and 24 h after injection.

systemic injection of MSCs led to preferential accumulation in the lungs [15], whereas portal injection resulted in retention in the liver [16]. The exact mechanism of entrapment of the MSCs in the capillary bed remains unknown, but the large size of the MSCs [15,17], their cellular attachment ability through expression of adhesion molecules [18,19] and appropriate flow characteristics [19] in the capillary bed, which favour endothelial adhesion, are probably key factors.

The actual number of MSCs remaining in the distal limb after i.a. administration could not be exactly determined, but both the calculation of the percent uptake and the relative height of the plateau on the time–activity curve were indirect quantification of MSC retention. The percent uptake underestimated the percent of MSCs remaining in the limb as the attenuation of the gamma rays by the limb itself underestimated the radioactivity present in the limb. In the current study, owing to the more diffuse distribution of the MSCs, the attenuation is probably even higher, since the osseous structures will also contribute. An exact attenuation correction is not possible because of the diffuse distribution of the MSCs, leading to different levels of attenuation depending on MSC location. For example, on the lateral images there is more attenuation of the radioactivity arising from the medial aspect of the limb than from the lateral aspect. An approximation can, however, be obtained by considering an average depth of attenuation. The diameter of a limb varies approximately from 4 to 10 cm from the metacarpus to the foot, so the centre of the limb can be estimated as approximately 3 cm deep. Considering an average depth of attenuation of 3 cm and using the attenuation equation with a soft tissue attenuation coefficient (0.153/cm), an attenuation of 37% is obtained [20]. This would suggest that if a 28% uptake is observed, 44% of MSCs are retained in the limb.

The relative height of the plateau of the time–activity curve is another way to estimate MSC retention, but the speed of injection can potentially modify the height of the peak. A slower injection will potentially lead to overestimation of the persistence of the MSCs. Based on this, and considering the initial median uptake of 28% and the median relative height of the plateau of 58%, it is likely that approximately half of the MSCs administered into the artery remained in the distal extremity initially.

The distal distribution of MSCs after i.a. injection in this study was similar to that previously observed with i.a. RLP [10]. However, the distribution to the carpus and proximal metacarpus was better in the previous study. In both studies the catheter was positioned in the same location, at the level of the chestnut. The catheter most commonly penetrated the median artery just proximal to the bifurcation of the radial artery, but the tip of the catheter commonly ended up advanced distal to the bifurcation. In the presence of the tourniquet it is likely that some of the MSCs were distributed through the radial artery to perfuse the carpus and proximal metacarpus, whereas in the absence of blood stasis, the MSC did not reach the radial artery. In the absence of a tourniquet, catheterising the artery more proximally, to ensure that the tip of the catheter would remain proximal to the origin of the radial artery, would probably help direct MSCs to lesions in the proximal metacarpal area. This would, however, be

challenging to achieve as the median artery is located deeper further proximally.

The limitation of poor or absent MSC distribution distal to the metacarpus identified with cephalic i.v. RLP [10] was overcome by the injection in the lateral palmar digital vein in this study, as all horses had uptake in the fetlock, pastern and foot areas. A limitation in the distribution was, however, the lack of uptake at the medial aspect of the distal limb. This is related to the vascular anatomy of the distal limb, but suggests that for diffuse distribution of MSCs through i.v. RLP to the entire distal limb both the lateral and medial palmar digital veins should be injected.

When comparing i.a. injection in the median artery and i.v. RLP through the lateral palmar digital vein for MSC administration to the distal limb, i.a. injection presented a much better distribution, both in terms of diffusion and homogeneity. The diffusion was again related to the vascular anatomy. The median artery provides blood supply to the entire distal limb, unlike the lateral palmar digital vein, which drains only the lateral aspect of the limb. The homogeneity of the distribution is most likely related to the final location of the MSCs. With i.a. injection the MSCs reached the capillary level, whereas MSCs injected with i.v. RLP probably remained in larger vessels, giving a more heterogeneous appearance. These differences suggested that the i.a. injection was more advantageous as it provided a more even tissue penetration. In order to perfuse the entire foot using the i.v. RLP technique, catheters may need to be placed in both the lateral and medial palmar digital veins. Alternatively, for a uniaxial lesion, i.v. RLP could be performed with a single catheter on the same side as the lesion; however, based on the pattern of distribution, the i.a. injection should still be preferred.

Regarding the comparison of percent uptake, the presence of the tourniquet on the i.v. RLP technique was obviously responsible for the higher initial uptake. However, it was interesting to observe that no significant difference in uptake was present at 24 h after administration. This correlated well with the observation that after 6 h, the persistence was higher after i.a. injection than after i.v. RLP. Intravenous RLP led to more MSCs present initially, but these MSCs were more likely to depart from the limb over time. This is also likely related to the actual location of the MSCs. Intra-arterially injected MSCs are more likely to be embedded in the capillaries than the MSCs injected with i.v. RLP, which remained in larger vessels.

No clinical complications were noted with either technique. Interestingly, no thrombi were observed ultrasonographically after i.a. injection, but ultrasonography revealed subclinical arterial thrombosis in the majority of limbs injected with i.v. RLP. These results and those from our previous study [11] suggest that the blood stasis itself is sufficient to create subclinical thrombi.

In conclusion, the 2 techniques investigated in this study are reliable and safe ways of administering MSCs to the equine foot. The i.a. injection would be the preferred technique based on the more homogeneous pattern of distribution and better long-term persistence. Also, although i.v. RLP has the advantage of being easier to perform, catheter placement in the lateral palmar digital vein does not perfuse the entire foot. The data presented here were obtained on horses under general anaesthesia. Further work is needed to assess the feasibility and efficiency of these techniques in a standing horse.

Authors' declaration of interests

Ethical animal research

Source of funding

Acknowledgements

Authorship

J. Trela was responsible for horse care, palmar digital vein catheterisation, tourniquet application, scintigraphic images acquisition, ROI count measurements, and redaction of the manuscript. M. Spriet was responsible for grant writing, study design, arterial catheterisation, MSC injection, scintigraphic images acquisition, uptake and persistence calculation, statistical analysis, figure preparation, and redaction of the manuscript. K. Padgett was responsible for culture and labelling of the MSCs. L. Galuppo was responsible for grant writing, study design, and manuscript revision. B. Vaughan was responsible for ultrasound evaluation and manuscript revision. M. Vidal was responsible for grant writing, supervision of MSCs culture and labelling, and manuscript revision.

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