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PII: S1090-0233(18)30379-4
DOI: https://doi.org/10.1016/j.tvjl.2018.07.004
Reference: YTVJL 5177

To appear in:

Accepted date: 13-7-2018

Please cite this article as: Laura Barrachina, Antonio Romero, Pilar Zaragoza, Clementina Rodellar, Francisco José Vázquez, Practical considerations for clinical use of mesenchymal stem cells: From the laboratory to the horse (2018), https://doi.org/10.1016/j.tvjl.2018.07.004

Review

Practical considerations for clinical use of mesenchymal stem cells: From the laboratory to the horse

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Highlights

- Equine practitioners should be aware of conditions that could affect the administration of mesenchymal stem cells (MSCs).
- Time, temperature and shipping media during MSC transport are critical points to slow down reductions in cell viability.
- Needles of 18G and 20G should be used to homogenise the MSC suspension, and to perform the injection, respectively.
- MSCs can be combined with other products such as hyaluronic acid or polysulphated glycosaminoglycans.
- MSCs should not be administered in combination with local anaesthetics, antibiotics or corticosteroids.

Abstract

Since the clinical use of mesenchymal stem cells (MSCs) for treating musculoskeletal injuries is gaining popularity, practitioners should be aware of the factors that may affect MSCs from tissue harvesting for MSC isolation to cell delivery into the injury site. This review provides equine practitioners with up-to-date, practical knowledge for the treatment of equine patients using MSCs. A brief overview of laboratory procedures affecting MSCs is provided, but the main focus is on shipping conditions, routes of administration, injection methods, and which commonly used

products can be combined with MSCs and which products should be avoided as they have deleterious effects on cells. There are still several knowledge gaps regarding MSC-based therapies in horses. Therefore, it is important to properly manage the factors which are currently known to affect MSCs, to further strengthen the evidence basis of this treatment.

Keywords: Horse; Mesenchymal stem cells; Practical considerations; Product combinations; Transport

Introduction

Mesenchymal stem cells (MSCs) are gaining popularity in equine practice for regenerative purposes, not only because of their potential for differentiation but also because of their trophic, anti-inflammatory and immunomodulatory abilities (da Silva Meirelles et al., 2009). In the horse, their most common application is the treatment of musculoskeletal injuries, which will constitute the main focus of this review. However, MSC-based therapies have also been explored for respiratory (Zucca et al., 2016), reproductive (Falomo et al., 2015) or ophthalmologic diseases (Sherman et al., 2017).

Notwithstanding reported beneficial effects and increasing clinical use of MSCs, their actual therapeutic efficacy is not yet entirely clear. In general, results of MSC treatment of equine tendinopathies have been more consistent than of joint pathologies (Colbath et al., 2017). Ultrasonographic and/or histopathologic improvements have been reported after MSC treatment of tendinopathies in experimental models (Caniglia et al., 2012; Romero et al., 2017) as well as in naturally occurring disease, with 77 to 98% of racehorses returning to racing with reinjury rates lower than 30% (Godwin et al., 2012; Van Loon et al., 2014). In equine osteoarthritis (OA) models, MSCs have

shown different results ranging from only a slight improvement (Frisbie et al., 2009) to a significantly improved outcome (Mokbel et al., 2011). In one retrospective study, 78% of horses with naturally occurring OA returned to work after MSC treatment (Broeckx et al., 2014a). Similarly, 76% of horses with different stifle injuries receiving MSCs after surgery returned to work, with the percentage of horses with meniscal injury returning to work being significantly higher than in previous studies using only arthroscopy (Ferris et al., 2014).

The wide variety of study designs, including different natural or experimental models as well as different treatment setups (e.g. time for treatment, MSC source or MSC number, etc.), precludes drawing definitive conclusions about actual MSC effectiveness. Furthermore, MSCs are often combined with other products or with surgical procedures, complicating the formulation of a conclusion on the role of MSCs. Although MSCs may be a promising treatment for equine musculoskeletal injuries, it is important to highlight that their actual therapeutic potential still remains unclear and that there are still several gaps in the knowledge to be investigated. Current knowledge of MSC therapies has been covered in other reviews (Colbath et al., 2017; Durgam and Stewart, 2017). The current review does not aim to report the efficacy of MSC and associated challenges, but offers practical guidelines to manage factors affecting the clinical use of MSCs.

Considerations ranging from laboratory procedures to shipping conditions and MSC administration may affect the clinical use of MSCs. Therefore, clinicians should be aware that MSCs are not a ‘traditional drug’, but a biological compound that must be handled carefully to ensure optimal administration. The aim of this review is to summarise the current knowledge about appropriate MSC management in clinical

practice, focusing on practical considerations to optimise the conditions in which MSCs are delivered to the equine patient.

Tissue harvesting for MSC isolation

In the horse, MSCs have been isolated from bone marrow (BM), adipose tissue (AT) (Ranera et al., 2011), peripheral blood (PB) (Dhar et al., 2012), synovial membrane and synovial fluid (Prado et al., 2015), amniotic membrane and fluid or umbilical cord (UC) blood and tissue (Iacono et al., 2012; Iacono et al., 2017), amongst others. Despite the wide variety of sources, not all of them are equally suitable for clinical purposes. Bone marrow and AT currently are the most extensively investigated sources for MSC isolation for clinical purposes (Colbath et al., 2017). Nevertheless, peripheral blood and perinatal sources are of raising interest because tissue harvesting is not invasive (Broeckx et al., 2014a, b; Tessier et al., 2015). Additionally, MSCs derived from perinatal sources present lower expression of immunogenic markers, potentially making them more suitable for allogeneic application (Tessier et al., 2015).

Equine BM and AT-MSCs properties have been compared quite extensively in vitro, with BM-MSCs showing higher chondrogenic (Vidal et al., 2008) and osteogenic (Toupadakis et al., 2010) potential. However, although MSCs from both sources display immunomodulatory properties, AT-MSCs seem slightly superior (Remacha et al., 2015) and elicit their regulatory effects through different mechanisms (Carrade Holt et al., 2014). Different properties shown in vitro by equine BM and AT-MSCs may be relevant for their clinical application, depending on the injury. However, only few studies have compared BM-MSCs and AT-MSCs in vivo. Iacono et al., (2015) reported beneficial effects of both BM-MSCs and AT-MSCs without significant differences between them in naturally occurring tendinopathies. Similarly, both treatments showed similar efficacy when surgically created meniscal defects were treated with scaffolds

loaded with either BM-MSCs or AT-MSCs (Gonzalez-Fernandez et al., 2016). Our group has compared equine BM-MSCs and AT-MSCs for treating experimentally induced tendon injuries. Although differences between treatments were relatively small, BM-MSCs resulted in a better outcome than AT-MSCs (Romero et al., 2017). In general, BM-MSCs are considered superior to AT-MSC for musculoskeletal therapy, which may be influenced by a larger number of scientific studies on BM-MSC (Schnabel et al., 2013).

Adipose tissue is usually harvested from the supra-gluteal subcutaneous area and BM can be collected from sternum or ilium with a Jamshidi needle. As aggregates formation may diminish cells recovery (Bastos et al., 2017), BM must be collected with an anticoagulant and the syringe gently agitated to ensure proper mixing. The preferred anticoagulant for BM collection is sodium heparin at 250-500IU/mL BM (Kasashima et al., 2011; Delling et al., 2012). No differences have been observed between BM-MSCs from sternum or ilium regarding proliferation, phenotype or differentiation (Adams et al., 2013; Lombana et al., 2015). The number of nucleated cells obtained from each location is similar in horses younger than 5 years (Adams et al., 2013) but lower in BM obtained from the ilium in older horses, in which iliac BM aspiration may be harder than sternal BM aspiration (Delling et al., 2012). Therefore, in animals older than 5 years, it is recommended to sample the sternum, whereas in animals younger than 5 years, the choice between sternum and ilium relies on individual preferences. The highest concentration of nucleated cells is contained in the first 5 mL of BM aspirate for both sampling locations (Adams et al., 2013).

Harvested tissue is sent to the laboratory for MSC isolation in containers at 4 ºC. Our laboratory has observed significantly lower numbers of colony forming units when BM is processed after 24 h of transport compared with isolation immediately after

aspiration, which could result in longer time required for expansion. Therefore, processing BM immediately after harvesting is preferred whenever possible (Ranera, 2012).

What happens in the laboratory?

Even though this review does not aim to describe laboratory techniques, it is important to understand the laboratory procedures performed once the sample for MSC isolation is received. Particular focus will be given to aspects potentially influencing MSC application.

MSCs isolation and culture

When MSCs are isolated from 'liquid' sources (BM, PB, UC-blood), the fraction of mononuclear cells is usually isolated by gradient centrifugation, whereas 'solid' sources (AT, UC-tissue) require enzymatic digestion. Subsequently, the mononuclear cell fraction is plated in tissue culture-treated plates to allow cell attachment while non-adherent cells will be removed with media replacements (Ranera et al., 2011; Tessier et al., 2015). Liquid harvests can also be directly plated mixed with culture media (Sharma et al., 2014) and MSCs can be isolated from solid tissues using explants techniques, although this results in lower MSC yields which can delay therapy (Gittel et al., 2013). After isolation, colonies of adherent cells appear within a few days and will progressively cover the plate bottom until they reach confluence. Cells are then enzymatically detached and reseeded in lower density to allow continuing expansion. This step is repeated each time cells become confluent and is known as a 'passage'. To complete a passage takes about 1 week, depending on technical and individual factors. MSCs are commonly applied at low passage to maintain stemness, i.e. passage 2-4, to obtain both an appropriate cell number and a homogeneous cell population (Colbath et al., 2017). For clinical application, the use of MSCs beyond passage 6-7 should be

avoided, as these cells become senescent, showing decreased proliferation and morphological abnormalities (Vidal et al., 2012). In conclusion, it takes about 2 to 3 weeks to obtain autologous cells ready for therapy. However, the required expansion time also depends on other points such as MSC proliferation potential, which may be lower in elderly patients (Choudhery et al., 2014), or the isolation protocol used (Bourzac et al., 2010; Gittel et al., 2013), etc. The process to obtain MSCs has been briefly outlined in Fig. 1.

The use of fetal bovine serum in the culture media

To provide the cells with nutrients and growth factors, fetal bovine serum (FBS) is commonly used to supplement MSC culture medium. This is a pivotal aspect for MSC clinical application as FBS is a xenogeneic compound that may generate an immune reaction (Sundin et al., 2007). Even if MSCs are rinsed exhaustively, cells may internalise some FBS-compounds. Mild inflammatory reaction may occur after intra-articular (IA) administration of both autologous and allogeneic MSCs in an equine healthy joint (Carrade et al., 2011; Pigott et al., 2013b; Ardanaz et al., 2016). This issue could be associated with xeno-contamination from the FBS, as the immune system may react against xeno-proteins internalised by MSCs (Sundin et al., 2007). Moreover, the animal might have been previously immunised because some xeno-proteins can be used for preparation of vaccines (Ohmori et al., 2005; Gershwin et al., 2012). Anti-FBS antibodies have been found in horses prior to MSC injection, but their titers were not modified after MSC administration (Owens and Kol, 2016). Internalisation of FBS-components by equine MSCs has been confirmed by using fluorescent labeled-FBS. Subsequent FBS-depletion was conducted by replacing the media with autologous serum-supplemented media over 48 h, demonstrating a reduction in intracytoplasmic fluorescence (internalised FBS-compounds) > 95% (Joswig et al., 2017). When

autologous MSCs (FBS-depleted or non-depleted) were administered into healthy equine joints, horses that received non-depleted cells showed adverse clinical reactions compared to those receiving FBS-depleted MSCs (Joswig et al., 2017). Therefore, replacing FBS-supplemented media with FBS-free media during the 48h prior to MSC administration is a suitable strategy to reduce the risk of joint flare. However, as autologous serum is less nutritional than FBS, cell viability may decrease during the depletion period, and further supplementation of medium may be needed (Joswig et al., 2017).

Several strategies have been developed to avoid the use of FBS (Table 1). The use of commercially available FBS-free media is still very limited in veterinary species and culturing equine MSCs in these media did not affect cell proliferation or phenotype, but did alter their immunomodulatory properties (Clark et al., 2016). The use of platelet lysate (PL) as FBS substitute is a highly interesting option. It is obtained by concentrating and subsequently lysing the platelet fraction from whole blood. This compound has been studied extensively for human MSC culture, but only few studies have been conducted with equine MSCs. Appropriate proliferation and differentiation of equine MSCs cultured on PL has been reported (Del Bue et al., 2007; Seo et al., 2013; Russell and Koch, 2016), but the effect on other properties of MSC, such as immunomodulation, clinical safety and the advantages over traditional FBS-culture need to be elucidated.

In conclusion, adverse reactions associated to FBS may occur during MSC administration and strategies have been developed to minimise the risk, but a widespread standardised technique is lacking.

Autologous vs. allogeneic MSCs

The use of allogeneic MSCs is subject to specific national regulations which are important for both clinicians and supplying companies. Nevertheless, allogeneic therapy has gained interest because it allows greater and more rapid cell availability. MSCs can be isolated from healthy donors to create cellular banks, thus enabling the use of well-characterised cells in the early phase of the injury, avoiding the aforementioned delay associated with autologous cells expansion. In human medicine, it also been suggested that allogeneic MSCs could also allow cellular therapy for elderly patients and for patients with genetic alterations or metabolic disorders, such as equine metabolic syndrome, which would preclude the use of autologous cells (Chen and Tuan, 2008; Zhang et al., 2015; Marycz et al., 2016).

MSCs have long been considered 'immune-privileged', but there is growing evidence for their possible recognition and elimination by the receptor immune system. Variable expression level of major histocompatibility complex (MHC) type I and II molecules and MHC-mismatch between donor and receptor may lead to cellular and/or humoral immune responses (Pezzanite et al., 2015; Berglund and Schnabel, 2016). MHC-I and MHC-II molecules expressed by donor MSCs could be directly recognised by recipient CD8+ or CD4+ T-cells, respectively. Direct recognition by naïve T-cells would also require costimulatory molecules such as CD40, CD80 or CD86 which are not expressed by MSCs in basal conditions. However, indirect recognition, which would not need costimulatory signals, may occur through internalisation of MHC-I and MHC-II fragments by the recipient antigen-presenting cells and subsequent presentation to B and T-cells, potentially generating immune memory that might limit repeated administration (Consentius et al., 2015). Furthermore, the exposure to inflammatory molecules can increase the expression of MHC-II and costimulatory molecules such as CD40 in equine MSCs, potentially increasing their immunogenicity. Nevertheless,

inflammatory stimuli also promote the MSC immunomodulatory profile, facilitating evasion of allogeneic cells to the immune system (Barrachina et al., 2017).

Immune targeting of MSCs may not only limit their effectiveness but might also compromise their safety. However, clinical implications are currently not clear. Single and repeated IA administration of allogeneic MSCs has been shown to be safe in both healthy equine joints (Carrade et al., 2011; Ardanaz et al., 2016) and joints with naturally-occurring disease (Broeckx et al., 2014a, b). Slight and transient joint inflammation detected after both autologous and allogeneic IA administration is usually self-limiting and spontaneously resolves within a few days (Pigott et al., 2013a, b; Ardanaz et al., 2016). Potential explanations for this reaction include the exquisite sensitivity of the equine joint and/or reaction to xeno-contaminants (Carrade et al., 2012). However, repeated IA injections of autologous or allogeneic MSCs after FBS-depletion produced higher nucleated cell counts in synovial fluid from the allogeneic group compared to the autologous group after the second injection, but significant differences were not demonstrated for other synovial or clinical parameters (Joswig et al., 2017).

Nevertheless, concerns about allogeneic MSCs should be taken into consideration and further research is needed to clarify them. Based on Schnabel et al. (2014), Berglund and Schnabel (2016) and Berglund et al. (2017), potential strategies to enhance safety, and thus effectivity, of allogeneic cells are summarised in Table 1.

From the laboratory to the clinic: Transport of equine MSCs

The three main variables during transport are shipping medium, temperature and duration, which are interconnected. For example, depending on the expected duration of transport, cells can be sent fresh or frozen, requiring different temperature and media.

Transport media

Transport media can be classified into two main types. Transport media requiring removal before administration are referred to as ancillary media. In contrast, excipient media are cell-carrier solutions supporting cell viability during transport, but these are unreactive, so they can be injected directly with MSCs. The disadvantages of ancillary media include the necessity of laboratory equipment to properly remove media and the risk of contamination during product manipulation outside the laboratory. MSCs should be rinsed extensively prior to administration, but nevertheless, xeno-contaminants may have already been internalised and viability can decrease during washes (Atouf, 2016; Williams et al., 2016a).

To slow down the reduction in viability during transport of fresh cells, one suggested strategy is to supplement transport media with serum. Autologous serum may not be available if the horse is far away, so allogeneic or xenogeneic compounds (i.e. FBS) would be required, thus constituting an ancillary medium. However, supplementation of phosphate buffered saline (PBS) with different concentrations of equine serum or FBS did not result in superior cell viability over PBS alone (Bronzini et al., 2012). Moreover, different biological compounds (BM aspirate, plasma, serum, platelet rich plasma [PRP]) as equine MSC-carriers were not superior to PBS for equine MSC viability up to 24 h (Garvican et al., 2014). Furthermore, after 24 h, cell viability decreased with all media, but particularly with the biological media, and proliferation was higher in PBS-shipped MSCs (Garvican et al., 2014). Therefore, biological products as MSC-carriers for transport are discouraged, although they could be therapeutically useful in combination with MSCs, as explained further in this review.

Since media supplemented with biological products or FBS did not provide significant advantages (Garvican et al., 2014) and ancillary media can present several disadvantages, serum/protein-free excipients have been recommended (Atouf, 2016;

Williams et al., 2016a). There are commercially available cell-preservation media that can be used as excipient for transport and administration of MSCs, such as HypoThermosol-FRS or CryoStor (BioLife Solutions). Their use in veterinary species is still limited, but appropriate equine MSC viability during transport and safe administration as excipients have been reported (Williams et al., 2016a).

Therefore, supplementing the transport media with biologic compounds or FBS does not seem advantageous and implies further manipulation, hence, increasing the risk of bacterial contamination. Therefore, isotonic saline solutions with neutral pH (7.2-7.4), such as PBS or lactated ringer's solution (LRS), are usually chosen as economic and practical cell-carriers that can be administered in combination with the cells.

Transport duration

There is consensus that fresh MSC transport should not take longer than 24 h, and that if transport duration exceeding 24 h is required, the most suitable option is to ship cryopreserved MSCs (Garvican et al., 2014). However, freezing media usually contain substances such as FBS or cryoprotectants that may not be suitable for in vivo administration (ancillary media). Broeckx et al. (2013 a, b) reported that cryopreserving equine MSCs in culture medium supplemented with only 10% DMSO preserved cell viability and direct administration after thawing was safe. Nevertheless, the recommended concentration for freezing under these conditions is 2 x 10^6 MSCs/mL (Broeckx et al., 2013a), so depending on the number of cells needed, the total volume may be too high, necessitating post-thawing concentration steps.

Transport temperature

Frozen cells should be shipped on dry ice or liquid nitrogen to maintain temperature around -80 ºC or -196 ºC, respectively. Studies have evaluated fresh MSC transport at 4 ºC, 37 ºC (body/culture temperature) and room temperature (RT, 20-22

ºC) in PBS or DMEM, either alone or supplemented with horse serum or FBS (Bronzini et al., 2012), or DMEM alone (Mercati et al., 2014). The general consensus is that 4 ºC is the most appropriate temperature for transport of fresh MSC, but one study reported that RT was superior (Bronzini et al., 2012) and may be preferred when the distance between the laboratory and the clinic is limited and transport duration is short. Since RT can vary considerably and it is difficult to keep an even temperature of 37 ºC during transport, 4 ºC seems the most practical temperature and can be provided using semen shipping containers.

Other transport variables

The type of container used and the MSC concentration in the shipping medium have been assessed in the interests of transport optimisation. The use of different plastic and glass containers did not show differences in cell viability after 24 h RT and differences were not observed between several MSC concentrations (5×10^6, 10×10^6 and 20×10^6 MSCs/mL) for transport of fresh (4 ºC) and cryopreserved cells (Espina et al., 2016). Plastic containers are commonly used for practical reasons and it is generally accepted that MSC concentration should be as low as possible to prevent cell aggregation. However, large volumes may not be suitable for clinical administration or may require further manipulation to concentrate the cells. Thus, cell concentrations ranging from 5×10^6 to 10×10^6/mL are generally used for shipping fresh MSCs (Garvican et al., 2014; Lang et al., 2017).

The main factors influencing equine MSC viability during transport are summarised in Table 2. Shipping frozen MSCs is most appropriate to limit reductions in cell viability; cell viability can be maintained at approximately 80% (Garvican et al., 2014), even up to 6 months (Broeckx et al., 2013a). However, further post-thaw manipulation may be required, and there can be associated disadvantages. In general,

shipping fresh equine MSCs in isotonic saline solution at 4 ºC for up to 24 h is considered ideal for administration after receiving the cells. However, in these conditions, MSC viability still decreases to around 70% (Bronzini et al., 2012; Garvican et al., 2014; Espina et al., 2016). Therefore, research is ongoing to further improve MSC shipping conditions.

Administration of MSCs for equine musculoskeletal injuries

Main routes for MSC administration

For single mild, focal lesions of tendons and ligaments, direct intra-lesional MSC injection by using real-time imaging guidance (i.e. ultrasonography) is recommended. In core lesions that cannot be easily accessed or when extensive/multifocal lesions are encountered, MSCs can be administered within the tendon sheath (if applicable) or by regional perfusion through an intravenous catheter (Schnabel et al., 2013). Intra-arterial administration is discouraged because of the risk of thrombosis (Sole et al., 2012). For joint pathologies such as osteoarthritis (OA) or meniscal injury, MSCs can be directly injected intra-articularly. For focal cartilage lesions, it would be recommended to retain MSCs into a scaffold and place them directly into the defect under arthroscopic guidance (Schnabel et al., 2013).

In most of the aforementioned situations, as well as in other situations not related to musculoskeletal injuries, MSC administration requires passage through a needle, which may affect cell viability, and therefore, the needle/catheter diameter should be taken into account. Aspiration and re-injection of equine MSCs using different gauge (G) needles, replicating the effect of re-suspending cells prior to injection, produced higher cell viability and a larger percentage of intact cells when 20G needles were used compared with a 25G-needle (Lang et al., 2017). Another study assessed the effect of aspirating and injecting equine MSCs through different diameter

needles. Needle diameter did not affect cell viability when injecting cells, but aspiration of MSC suspension with needle diameters $\leq$ 20G led to decreased cell viability, which may have been due to negative pressure during aspiration (Williams et al., 2016b). Therefore, a negative effect on equine MSCs passing through a needle is mostly produced during aspiration rather than during injection, so 18G-needles or larger should be used to aspirate cell suspension from the shipping vial into the syringe, whereas a 20G-needle/catheter is recommended for injection. Moreover, the injection of the MSC suspension should be performed slowly to avoid excessive cellular stress.

How many cells, how many times and when?

These questions have been addressed in other reviews (Schnabel et al., 2013; Monteiro et al., 2015), so only a brief overview is provided here. To date, there are no 'dose-response' studies clarifying optimal MSC number for equine musculoskeletal injuries. Most of the available literature on equine soft tissue lesions (mostly tendons) reported MSC numbers ranging between 10×10^6 and 30×10^6, with 10×10^6 cells being most commonly used (Schnabel et al., 2009; Godwin et al., 2012). Furthermore, the current dose recommendation for equine OA by direct IA injection is 20×10^6 MSCs (Schnabel et al., 2013; Zayed et al., 2018).

There are no studies clarifying the most appropriate moment to apply MSCs. The optimal therapeutic window is considered to be during the subacute phase of tissue repair, when inflammation has decreased, and scar tissue formation is still limited (Koch et al., 2009). Higher reinjury rates of tendon lesions have been observed when MSC therapy was delayed (Richardson et al., 2007, Godwin et al., 2012). In most studies, autologous MSCs are administered in tendon lesions within 2-4 weeks post-injury (Caniglia et al., 2012; Carvalho Ade et al., 2013), while some studies have described even earlier administration (Schnabel et al., 2009; Romero et al., 2017). It

should be noted that such an early administration of autologous cells was only possible because MSCs were isolated before the experimentally induced injury, which would have limited clinical applicability unless autologous MSCs were cryopreserved prior to the development of pathology, or allogeneic MSCs were used. At least in the authors' experience, equine practitioners often reserve MSC-therapy as a 'last chance' option once all other conventional treatments have failed. Consequently, tissue damage may be severe and the prognosis may be poor regardless application of MSCs. Therefore, it may be advantageous to choose MSC therapy in an earlier phase of the injury.

The safety of repeated MSC injections has been assessed in the horse (Ardanaz et al., 2016; Joswig et al., 2017), but in terms of efficacy, most studies have focused on a single injection. However, repeated administrations have shown benefits in other animal models, such as a model of porcine meniscal injury (Hatsushika et al., 2014). Generally, it is recommended that horses are checked 30 days after MSC administration, when a second dose can be administered if the improvement is less than 50% (Schnabel et al., 2013).

What products can we combine with MSCs?

Equine MSCs can be administered suspended in isotonic pH neutral solution such as PBS or LRS, as provided by the laboratory for transport. As mentioned above, MSCs can also be combined with biological products such as PRP, BM-supernatant (BMS) or autologous conditioned serum (ACS) etc. There is no evidence of superior efficacy when combining MSCs with these products, or whether any additive or synergistic effects exist. Empirically, equine MSCs are often combined with PRP or BMS for tendon/ligament injuries and with ACS for joint pathologies, but this merely depends on the clinician's preferences (Schnabel et al., 2013).

For IA use, it is possible to combine equine MSCs with injectable gels such as hyaluronic acid (HA) or polysulphated glycosaminoglycans (PSGAG), but the combination with polyacrylamide hydrogel (PAAG) has been shown to decrease cell viability up to 50-70% within 24-48 h in vitro, possibly due to the lower pH of PAAG (Broeckx et al., 2013a). If MSCs are combined with HA for IA injection, the current recommendation is to use 20 x 10^6 MSCs in 22mg HA (3 x 10^6 Da) (Schnabel et al., 2013), which does not affect equine MSC viability (Bohannon et al., 2013). Moreover, the HA concentration should not exceed 10 mg/mL because higher concentrations might interfere with equine MSC migration (Broeckx et al., 2013a). To prevent joint flare, one dose of non-steroidal anti-inflammatory medication is recommended prior to MSC administration (Ferris et al., 2014); a bandage should be applied for 24-48h afterwards.

It is possible to suspend equine MSCs in biological or gel products if used immediately, but clotting may occur when products are combined in one syringe (particularly with PRP) and this can preclude injection. Therefore, mixing cells and other products should be avoided in one syringe, but they can be administered through the same needle sequentially (Schnabel et al., 2013).

Which products should not be combined with MSCs?

Sedation and regional anaesthesia are commonly performed to allow musculoskeletal treatments in horses. The effects of drugs used for these purposes on equine MSCs have been evaluated in vitro by estimating the amount that would come in contact with MSCs in vivo after IV administration (sedative drugs), or when performing regional anaesthesia (local anaesthetics). Romifidine, detomidine and butorphanol did not significantly affect cell viability, but xylazine slightly decreased viability (Edmonds et al., 2017). Even if this effect was slight, we recommend that xylazine is avoided when MSCs are administered.

Local anaesthetics used for perineural blockade do not affect equine MSC viability, as in vitro studies by Edmonds et al. (2017) have shown that the concentration of mepivacaine that would reach equine MSCs after a perineural block did not have negative effects. However, when equine MSCs were exposed to mepivacaine and procaine at higher concentrations, mimicking IA use, almost 90% of cells died within 3 h (Broeckx et al., 2013a). Therefore, the administration of MSCs after the injection of local anesthetics is strongly discouraged.

In some circumstances, antimicrobial drugs (i.e. aminoglycosides) may be administered prophylactically together with another therapeutic agent. However, in combination with MSCs, this procedure is not recommended, as in vitro exposure to therapeutic concentrations of gentamicin or amikacin have been shown to lead to a decrease in MSC viability of > 95% within 45 min to 2 h (Bohannon et al., 2013). Furthermore, even much lower concentrations of these drugs may induce changes in the gene expression of equine MSCs, possibly affecting their function (Parker et al., 2012).

Regarding corticosteroids, in vitro research has shown that therapeutic doses of methylprednisolone and triamcinolone decrease cell viability in equine MSCs, with the most marked effect for methylprednisolone (Edmonds et al., 2017). Therefore, corticosteroids and MSCs should not be used together, and should even not be administered with a short interval. Table 3 summarises which products can be combined with MSCs and which should be avoided for treating equine musculoskeletal injuries.

Conclusions

Mesenchymal stem cells have the potential to improve the care of equine patients. Although our understanding of many aspects of this therapeutic modality is limited, there is some published research regarding specific conditions affecting the use of MSCs. This review has summarised the manageable factors in each step of the

process, to optimize MSC administration. These practical considerations can contribute to building a stronger basis of clinical evidence, but further research is warranted to improve evidence-based strategies for MSC-based therapies in horses.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of paper.

Acknowledgements

The authors wish to acknowledge the Ministerio de Economía y Competitividad, España (AGL2008-02428/GAN and AGL2011-28609) for research support.

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Table 1 Overview of the process for clinical administration of mesenchymal stem cells (MSCs) for treating equine musculoskeletal injuries, emphasising critical factors and current recommendations and alternatives.

Step	Pivotal points	Recommendations / Alternatives	References
Tissue harvesting	Bone marrow aspiration	• Horses <5 years: ilium or sternum • Horses >5 years: sternum • First 5 mL are the richest in nucleated cells	Delling et al., 2012; Adams et al., 2013; Lombana et al., 2015
	Transport to lab	• 4 ºC (up to 24h) • Straight isolation after harvesting is preferred	Ranera, 2012; Bastos et al., 2017
MSC isolation and expansion	Xeno-contaminants in culture medium (i.e. fetal bovine serum)	• FBS-depletion • Commercial fetal bovine serum-free media • Platelet lysate	Del Bue et al., 2007; Seo et al., 2013; Russell and Koch, 2016; Joswig et al., 2017
Choosing between autologous /allogeneic	Major histocompatibility complex expression level and compatibility (beware of regulatory aspects)	• Select MSCs with low major histocompatibility complex expression • Study major histocompatibility complex matching between donor and receptor • Diminish expression of major histocompatibility complex	Schnabel et al., 2014; Berglund and Schnabel, 2016; Berglund et al., 2017
Transport	Shipping media, temperature, duration	(See Table 2) • Fresh cells: phosphate buffered saline, 4 ºC, up to 24h • Frozen cells: + dimethyl sulfoxide, dry ice, >24h	Bronzini et al., 2012; Broeckx et al., 2013a, b; Garvican et al., 2014; Mercati et al., 2014
Injection of MSCs	Route of administration	• Tendon/ligament ○ Discrete focal lesion: intra-lesionally ○ Extensive/multifocal lesion: sheath, regional perfusion • Joints ○ Osteo-arthritis, menisci: arthrocentesis ○ Focal cartilage defect: scaffold (arthroscopy)	Sole et al., 2012; Schnabel et al., 2013; Monteiro et al., 2015
	Needle diameter	• >18G for resuspension and loading into syringe • 20G for injection	Williams et al., 2016b; Lang et al., 2017
	Combination of MSCs with other products	(see Table 3) • Avoid intra-lesional local anesthetics, antibiotics and corticosteroids	Parker et al., 2012; Bohanon et al., 2013; Broeckx et al., 2013a; Edmonds et al., 2017

Table 2 Recommendations for mesenchymal stem cells (MSC) transport focusing on the shipping medium, the temperature during transport and the duration of transport (Bronzini et al., 2012; Broeckx et al., 2013a, b; Garvican et al., 2014; Mercati et al., 2014; Sole et al., 2012; Schnabel et al., 2013; Monteiro et al., 2015; Atouf, 2016, Williams et al., 2016a)

	Shipping medium		**Temperature**	**Duration**
		Manipulation required?		
Fresh MSCs	Ancillary: + fetal bovine serum, + allogeneic serum	Yes, to remove media and wash	Transport: 4 ºC Only transit: room temperature	Up to 24h
	Excipient: phosphate buffered saline, saline, Lactated Ringer's Solution (+/- autologous serum), commercial media	No, straight injection is possible		
Frozen MSCs	Ancillary: + fetal bovine serum, potentially toxic/high concentration cryoprotectants	Yes, to remove media and wash	-80 ºC (dry ice)	Preferable if longer than 24h
	Excipient: +10% dimethyl sulfoxide	Maybe, to concentrate cell suspension		

Table 3 Summary of products which can and cannot be combined with mesenchymal stem cells (MSCs).

Can we use MSCs with …?	**Yes**	**No**	**References**
Sedative agents	Alpha 2-agonist (romifidine, detomidine) + butorphanol IV	(Avoid xylazine)	Edmonds et al., 2017
Local anaesthetics	For regional blocks (tendon/ligament treatment) (mepivacaine)	Intra-articular treatment (mepivacaine, procaine)	Broeckx et al., 2013a; Edmonds et al., 2017
Injectable gels (intra-articularly)	Hyaluronic acid, polysulphated glycosaminoglycans	Polyacrylamide gel	Bohannon et al., 2013; Broeckx et al., 2013a; Schnabel et al., 2013
Biologic products	Platelet rich plasma, bone marrow supernatant, autologous conditioned serum (nor additive nor synergic effects have been proven)		Schnabel et al., 2013; Garvican et al., 2014
Antibiotics (intra-lesional)		Gentamicin, amikacin	Parker et al., 2012; Bohannon et al., 2013
Corticosteroids (intra-lesional)		Methylprednisolone, triamcinolone	Edmonds et al., 2017

Figure legend

Fig. 1. Overview of the process to obtain equine mesenchymal stem cells (MSCs) from tissue harvesting to laboratory procedures to isolate MSCs (RT, room temperature) (Ranera et al., 2011, 2012; Vidal et al., 2012; Tessier et al., 2015, Colbath et al., 2017).

Figr-1