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Guidelines to Optimize Survival and Migration Capacities of Equine Mesenchymal Stem Cells

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Abstract

Background: Although mesenchymal stem cell (MSC) therapy is commonly being used in veterinary medicine, no specific guidelines are described to guarantee an optimal cell survival and migration. However, this is indispensable in order to assure an optimal clinical use.

Methods: In the present study, equine peripheral blood (PB)-derived MSCs were isolated and characterized. In order to determine the optimal parameters for long-term cryopreservation, the effects of different dimethyl sulfoxide (DMSO) concentrations (5-20%) and thawing methods (temperature-controlled or hand-thawed) on MSC viability were evaluated by means of trypan blue staining. Afterwards, MSC storage was evaluated for 12 months using only 10% of DMSO as a cryoprotectant without rate-controlled freezing. Then, the influence of frequently used anaesthetics and injectable gels on MSC viability was assessed. Finally, the migration capacity of MSCs through hyaluronic acid at different concentrations was assessed.

Results: Firstly, we demonstrated that the DMSO concentration and the thawing method had no considerable influence on MSC viability within the 4 hour evaluation after thawing. Secondly, anaesthetics were highly cytotoxic and only 10% of the MSCs survived 3 hours of their presence. Hyaluronic acid- and glycosaminoglycan-based gels were compatible with MSCs and allowed a high cell survival (more than 90%) within 48 hours of follow-up. However, in a polyacrylamide gel, approximately 80% of the MSCs died within 48 hours. Finally, the concentration of the gel influenced the migration capacity of the MSCs. Indeed, only 60% of the MSCs in 20 mg/ml of hyaluronic acid attached to the underlying surface within 48 hours, whereas almost 100% of the MSCs in 10 mg/ml of the same substance were able to migrate through the gel.

Conclusion: The present study reports practical guidelines for the clinical application of equine PB-derived MSCs.

Keywords: Stem cells; Viability; Migration; DMSO

Introduction

In 1970, Alexander Friedenstein [1] was the first to evidence the presence of non-hematopoietic cells in the bone marrow that were capable of self-renewal and osteogenic differentiation. These cells were typed as mesenchymal stem cells (MSCs) because of their ability to differentiate into cells of the mesodermal germ layer [1]. Since the discovery of MSCs, their potential use in regenerative medicine has been studied with increasing interest. The possibility of functional tissue recovery after injury is probably the main reason why MSC therapy is frequently being used in horses, which are athletic animals at high risk of musculoskeletal injuries. However, no standardised guidelines currently exist to inform veterinarians on the appropriate handling and clinical applications of MSCs.

It has been reported that fresh equine PB-derived MSCs dramatically decline in cell number after 12 hours of transport and have a higher risk to become senescent after 24 hours of transport [2]. In this regard, the use of frozen samples may enhance the viability of the product. Nevertheless, frozen samples contain low concentrations of the cryoprotective substance dimethyl sulfoxide (DMSO). Although the safe clinical use of frozen equine MSCs has been described before [3-5], DMSO can have a cytotoxic effect on cells after thawing [6]. We therefore wanted to determine the effect of different freezing and thawing protocols on equine PB-derived MSC viability, to enable an optimal method to be developed for standardised clinical applications.

In addition, MSC injection after intra-articular anaesthesia has been performed in order to localize the area which causes the lameness. Besides an antiproliferative effect at a very low dose (100 µM) [7], there are currently no reports on the influence of anaesthetics on equine MSCs which are subsequently injected. In this study we determined the effect of two commonly used anaesthetics on MSC viability.

Prior to transplantation MSCs are often suspended in a carrier. Several carriers, such as phosphate buffered saline (PBS) [8], hyaluronic acid [9], as well as fibrin gels [10,11] have been reported in musculoskeletal stem cell transplantations in veterinary research. In the field, veterinary practitioners are mainly combining MSCs with substances containing extracellular matrix components, such as hyaluronic acid [12]. The main limitation, however, is that each substance has its own visco-elastic properties, pH and osmotic value, all of which can influence MSC viability and migration capacities, and therefore, potentially influence the clinical outcome of MSC therapy.

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Additionally, it has been reported that even within the same carrier (fibrin gel) the dilution has an effect on the migration of MSCs [13]. For all the aforementioned reasons, the present *in vitro* study also describes the effects of different injectable gels on the viability and migration capacity of equine PB-derived MSCs.

Materials and Methods

Isolation and characterization of equine peripheral blood (PB)-derived mesenchymal stem cells (MSCs)

In total 50 ml of blood was collected in sterile EDTA tubes from the *vena jugularis* of a 4-years-old donor gelding for mesenchymal stem cell (MSC) isolation and characterization as previously described by our group [5,14]. At 60% confluency the cells were trypsinized with 0.25% trypsin-EDTA and subcultured until passage 3 (P3) in expansion medium, consisting of low glucose DMEM (Gibco), 20% foetal bovine serum (Gibco) and 1% antibiotics – antimycotics (Sigma). The local ethical committee approved the experimental design (EC_2012_001).

Freezing and thawing

At P3, the cells were trypsinized and resuspended in 1ml of DMEM low glucose with 5, 7, 10 or 20% of dimethyl sulfoxide (DMSO, Sigma). No animal serum was used in the freezing protocols in order to reduce the risk of disease transmission in case of a clinical application. At this point 2×10^6 MSCs were frozen overnight in an isopropanol chamber at -80°C to produce a controlled freezing rate. The samples were then stored at -80°C for 1 week prior to thawing in a temperature-controlled warm water bath at 37°C or in the palm of one's hand. Viability was only assessed at the earliest time points after thawing (0.5, 1, 2 and 4 hours) because it would never take more than 4 hours for veterinary practitioners to prepare the injection site.

For longer term viability assays 2×10^6 MSCs were frozen without rate-control, but this time with the most suitable DMSO concentration (as determined by the aforementioned experiment) and viability was evaluated at 6, 8, 10 and 12 months storage at -80°C. Three samples per circumstance were evaluated.

Under field circumstances, rapid thawing at 37°C is not always possible. Therefore, the effect of thawing MSCs with different DMSO concentrations by hand was compared to using a temperature-controlled warm water bath at 37°C. Since there were no considerable differences between both thawing methods, cells were thawed in a temperature-controlled warm water bath at 37°C for the long-term viability evaluation.

Anaesthetics

1ml of DMEM with 0.5×10^6 PB-derived MSCs at P3 were added to 2 ml of different commercially available, commonly used anaesthetics in equine medicine: 20 mg/ml Scandicaine® (Mepivacaine Hydrochloride) with or without 5 µg/ml Adrenaline and 40 mg/ml Procaine® (Procaine Hydrochloride). In order to mimic the *in vivo* dilution and absorption of the anaesthetic after intra-articular injection, each product was *in vitro* diluted 1:5 with phosphate buffered saline (PBS) 1x before adding the MSCs. The control sample contained 1ml of DMEM with MSCs and 2 ml of PBS 1x.

Injectable gels

1 ml of DMEM with 0.5×10^6 PB-derived MSCs at P3 were resuspended in 2 ml of hyaluronic acid at 10 mg/ml (Ostenil® or Adant®) or 20 mg/ml (Ostenil® +), in 5 ml of 100 mg/ml polysulphated glycosaminoglycan (Adequan®) or 2 ml of 25 mg/ml polyacrylamide (Arthramid®). All gels were ready to use and all concentrations and volumes were used as indicated by the manufacturer for clinical application. As a control, 1 ml of MSCs was diluted in 2 ml of expansion medium.

Viability staining and migration assay

For all experiments 25 µl of cell suspension was diluted in 25 µl of trypan blue (Gibco) in order to perform a viability staining at different time points (0, 2, 4, 6 and 48 hours). The average of four fields of a Burker counting chamber was used to calculate the percentage of dead and alive cells. In order to assess the migration capacity of PB-derived MSCs individual wells of a 24 well plate were filled with the gels or with expansion medium as a positive control. A volume of 0.5 ml of PB-derived MSCs in DMEM was gently layered above the filled wells. The concentration of floating cells in the different wells was calculated at different time points (0, 2, 4, 6 and 48 hours). Pictures were taken at these time points in order to visualize the migration process and correlate the floating cells with the migration process.

Results

Isolation and characterization of equine peripheral blood (PB)-derived mesenchymal stem cells (MSCs)

After 17 days the first spindle shaped cells were noticed in the culture flasks and at 21 days after isolation the cells were trypsinized at approximately 60% confluency. The characterization experiments revealed that the isolated cells fulfilled all the requirements to be typed as MSCs according to the proposed guidelines by Dominici et al. [15].

Dimethyl sulfoxide (DMSO) concentration and duration of freezing has some effect on PB-MSC viability

It has been reported that frozen equine PB-derived MSCs possess the same stem cell characteristics as freshly isolated cells [16]. In this regard, freezing enhances the shelf-life of MSC samples. From the different dimethyl sulfoxide (DMSO) concentrations tested, the cells frozen in 10% DMSO showed the highest viability after maintaining for 1 week at -80°C (Figure 1). When evaluating the samples after 6, 8, 10 and 12 months of storage in 10% of DMSO, our results show that MSC viability decreased gradually down to 70% after 12 months of storage (Figure 2A). Using a rate-controlled freezing process displayed a similar MSC viability at 6 months after storage (data not shown).

Thawing in the field is usually performed by keeping the sample in the palm of one's hand. In order to compare the effects of thawing cells in field and laboratory conditions, the MSC viability was assessed after thawing in a temperature-controlled warm water bath and in the palm of the hand. In this regard, these preliminary experiments revealed that viability decreased over time due to the cytotoxic effect of DMSO, which was not related to the thawing method or the DMSO concentration (Figure 1). However, 4 hours after hand-thawing, cell viability was slightly higher in the 7 and 20% DMSO samples than when thawed in a water bath (Figure 1). Still, this does not implicate that hand-thawing is superior to temperature-controlled thawing.

Exposure of PB-MSCs to anaesthetic has a pronounced effect on cell viability

Approximately 30% of the MSCs died within the hour after adding MSCs to a Mepivacaine-based anaesthetic (Figure 2B). In a Procaine-

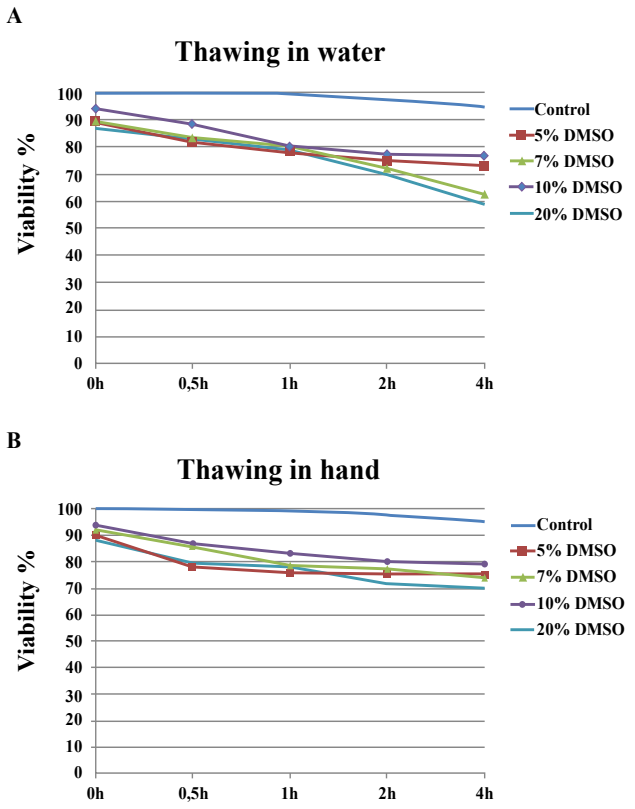

Figure 1: Influence of dimethylsulfoxide (DMSO) percentage and thawing method (temperature-controlled water bath = **A**; hand-thawing = **B**) on mesenchymal stem cell (MSC) viability (Y-axis) at different hours (h) after thawing (X-axis). The control represents fresh cells resuspended in 1 ml of DMEM.

based anaesthetic more than 40% of the cells died within the hour (Figure 2B). At 3 hours after adding MSCs to the anaesthetics, most (± 90%) of the MSCs had died (Figure 2B).

Different carriers have different effects on MSC viability

Viability staining revealed that hyaluronic acid (Ostenil® and Adant®) and glycosaminoglycan (Adequan®) gels had no noticeable cytotoxic effect on the MSCs. Indeed, the viability did not drop below 90% within 48 hours after addition of the MSCs to the commercially available gels (Figure 3A). The polyacrylamide gel (Arthramid®) on the other hand, had a negative effect on MSC viability. Almost half (44%) of the MSCs died within 24 hours and 77% died within 48 hours after adding the MSCs to the Arthramid® gel (Figure 3A).

Migration assay

Approximately 60% of the control MSCs migrated through the expansion medium within 4 hours, 90% attached within 24 hours and 100% within 48 hours (Figure 3B). When seeding the MSCs in a 10 mg/ml hyaluronic acid (Ostenil®)-covered well, within 24 hours 70% of the MSCs attached to the underlying surface and 98% within 48 hours (Figure 3B). However, when doubling the concentration of hyaluronic acid to 20 mg/ml (Ostenil® +), the MSCs had substantially more difficulties to migrate through the gel. Indeed, only 45% of the cells reached the underlying surface within 24 hours and 65% within 48 hours (Figure 3B). However, the reduced migration did not affect the MSC viability, which was still 92% at 48 hours after seeding. Figure 4 represents MSC attachment after migration through the different substances.

Discussion

In the present study, different dimethyl sulfoxide (DMSO) concentrations did not markedly influence equine peripheral blood (PB)-derived mesenchymal stem cell (MSC) viability within 4 hours after thawing. In addition, temperature-controlled thawing did not influence the cell viability in comparison to hand-thawing. In this regard, it has been described that thawing human peripheral blood stem cells (PBSCs) at 20°C or 37°C did not influence cell recovery or viability [17,18]. Therefore, we suggest that hand thawing is a suitable method to be used in the field.

In agreement with another report on cryopreservation of human PBSCs, our results show that 10% of DMSO is sufficient to prevent cell death as a sole cryoprotectant without rate-controlled freezing [19]. Moreover, 70% of the frozen MSCs survived the 1 year freezing process at ultralow temperatures. Consistent with a previous report on human hematopoietic progenitor cells stored for more than 9 years in liquid nitrogen [20], our current study showed no difference in short-term survival after storage with either 5% or 10% of DMSO.

In the field, local anaesthesia is usually performed in order to localize the origin of the lameness. With frozen samples, veterinarians

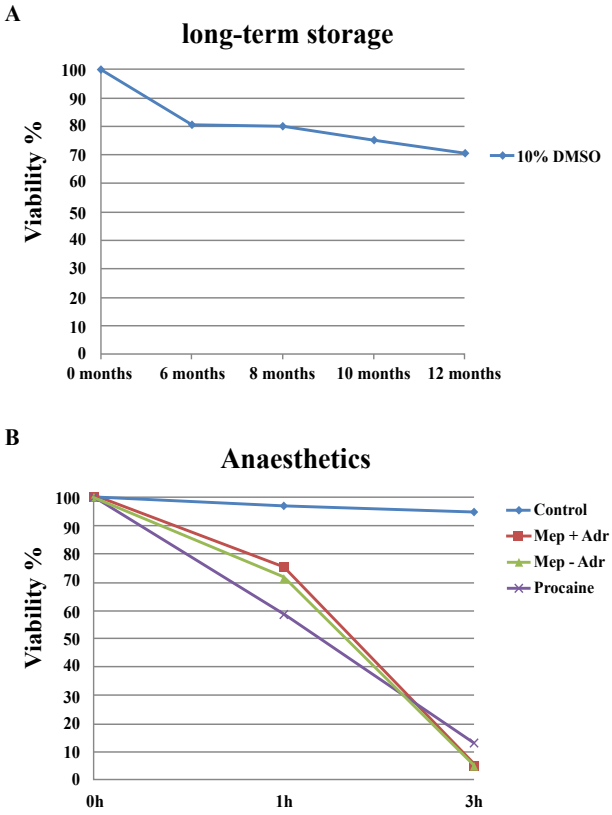

Figure 2: Mesenchymal stem cell (MSC) viability (Y-axis) after freezing with 10% DMSO and at different time points (X-axis) immediately after thawing **(A)**. The influence of different anaesthetics on MSC viability was tested at different hours (h) (X-axis) after mixing the MSCs with the following substances: Mepivacaine (Mep) with (+) or without (-) Adrenaline (Adr) and Procaine **(B)**. The control represents MSCs in 1 ml of DMEM diluted with 2 ml of PBS 1x.

are tempted to immediately treat the injured joint. However, our results show that exposure of MSCs to all of the anaesthetics tested resulted in significant cell death. Although the concentrations of anaesthetics to which the cells are exposed to *in vivo* may be lower than those used in this *in vitro* study, it does suggest that the use of anaesthetics immediately before MSC therapy should be avoided.

In certain circumstances, the use of injectable gels has been reported to act as a carrier for the MSCs [9,12,21]. After evaluating MSC viability in different injectable gels, it became clear that the polyacrylamide gel was not compatible with MSCs as it resulted in significant cell death. Further analysis of the different gels showed that the acidified pH of the polyacrylamide gel was probably at the basis of this observation (6.2 in comparison to the neutral pH of the other gels). Indeed, it has been reported that pH drop caused by acidified substrates decreased cell viability of equine BM-derived MSCs [12].

This study also showed that doubling the hyaluronic acid concentration - which increased the density of the gel - disturbed the migration process of the MSCs. This resulted in a lower surface attachment *in vitro* and may cause a decrease in cell integration *in vivo*. In this regard, it has been described that there was no evidence of any clinical or histological significant improvement in the equine medial femoro-tibial joints injected with bone marrow (BM)-derived MSCs in a 22 mg hyaluronic acid scaffold [9]. In contrast with Mcllwraith's [21] study, it has been reported that carpal joint arthrosis in donkeys improved clinically as well as histologically after treatment with BM-derived MSCs resuspended in 3 ml of hyaluronic acid. Moreover, the MSCs were able to integrate in the cartilage, indicating that the MSCs participated in the healing process of the damaged tissue. Unfortunately, the exact concentration of the hyaluronic acid gel was not provided in the second study. However, we have clearly demonstrated that the concentration of hyaluronic acid does influence MSC migration and this should be taken into account when considering the clinical application of the cells.

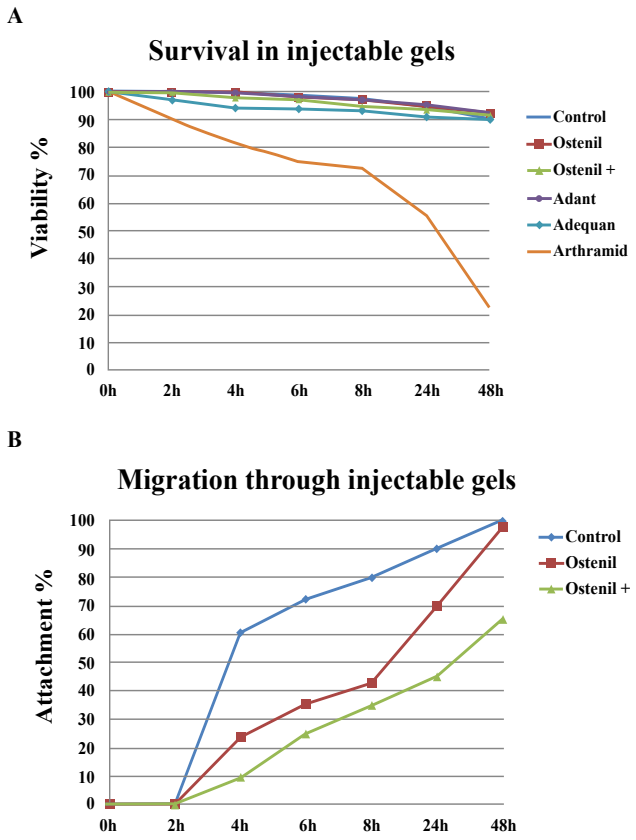

Figure 3: Influence of different injectable gels on mesenchymal stem cell (MSC) viability **(A)** and migration capacity **(B)** at different hours (h) after adding MSCs to the gels (X-axis). The controls represent MSCs in 1 ml **(A)** or 0.5 ml **(B)** of DMEM diluted with 2 ml **(A)** or 1 ml **(B)** of expansion medium.

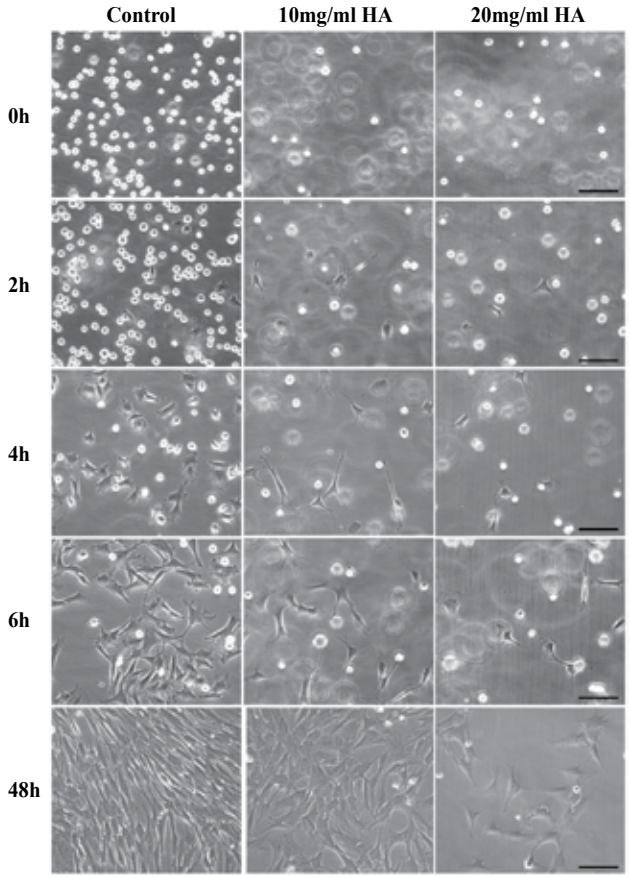

Figure 4: Light microscopic images of mesenchymal stem cell (MSC) migration at different hours (h) through expansion medium (control), 10 mg/ml hyaluronic acid and 20 mg/ml hyaluronic acid. Scale bars represent 50 µm.

The findings of this current study demonstrate that the variable methods used during clinical application of MSCs in the veterinary field may have significant effects on the cells themselves, which in turn is likely to affect their clinical efficacy. Additional research to standardise the protocols used would therefore be of benefit.

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Author Disclosure Statement

The authors MS and JS declare competing financial interests in Global Stem cell Technology (GST).

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