Original Article

Bone marrow-derived multipotent mesenchymal stromal cells from horses after euthanasia

Carmen Schröck*, Carina Eydt†, Florian Geburek‡, Lena Kaiser*, Felicitas Päbst§,¶, Janina Burk§,¶, Christiane Pfarrer† and Carsten Staszyk*

*Institute for Veterinary Anatomy, -Histology and -Embryology, Justus-Liebig-University, Giessen, Germany, †Institute of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany, ‡Clinic for Horses, Justus-Liebig-University, Giessen, Germany, §Translational Centre for Regenerative Medicine (TRM), University of Leipzig, Leipzig, Germany and ¶Faculty of Veterinary Medicine, Large Animal Clinic for Surgery, University of Leipzig, Leipzig, Germany

Abstract

Allogeneic equine multipotent mesenchymal stromal cells (eMSCs) have been proposed for use in regenerative therapies in veterinary medicine. A source of allogeneic eMSCs might be the bone marrow from euthanized horses. The purpose of this study was to compare *in vitro* characteristics of equine bone marrow derived eMSC (eBM-MSCs) from euthanized horses (eut-MSCs) and from narcotized horses (nar-MSCs). Eut-MSCs and nar-MSCs showed typical eMSC marker profiles (positive: CD44, CD90; negative: CD11a/CD18 and MHCII) and possessed tri-lineage differentiation characteristics. Although CD105 and MHCI expression varied, no differences were detected between eut-MSCs and nar-MSCs. Proliferation characteristics did not differ between eut-MSCs and nar-MSCs, but age dependent decrease in proliferation and increase in MHCI expression was detected. These results suggest the possible use of eut-MSCs for therapeutic applications and production of commercial available eBM-MSC products.

Keywords: equine stem cells, flow cytometry, horse, proliferation assay, regenerative therapy.

Correspondence: Carsten Staszyk, Institute for Veterinary Anatomy, -Histology and -Embryology, Justus-Liebig-University, Giessen, Germany.
E-mail: carsten.staszyk@vetmed.uni-giessen.de

Introduction

Equine bone marrow-derived multipotent mesenchymal stromal cells, obtained from live horses, are the focus of multiple experimental and clinical studies evaluating their characteristics with regard to their use in regenerative therapies (Arnhold *et al.* 2007; Brehm 2008; Cortes *et al.* 2013; Renzi *et al.* 2013; Smith *et al.* 2013). As far as the authors are aware, however, there have been no experimental studies reported which use eBM-MSCs obtained from euthanized horses. It is widely accepted that multipotent mesenchymal stromal cells (MSCs) selected for therapeutic use express MSC-typical cell surface markers such as CD44, CD90 and CD105, and provide extensive proliferative capacity *in vitro* (Iacono *et al.* 2012b; Spaas *et al.* 2013). For most therapeutic approaches autologous sternal bone marrow is harvested in order to isolate and expand eBM-MSCs. Although the application of autologous MSCs avoids the risk of immunorejection of the applied cells, there are several problems associated with obtaining autologous eBM-MSCs. First, the MSCs may be altered by disease status and treatment with pharmaceutical products. Second, the harvesting procedure bears the risk of thoracic and cardiac punctures (Jacobs *et al.* 1983; Durando *et al.* 2006). Third, the production of an injectable autologous eBM-MSC product is a time consuming procedure that takes up to five weeks (Brems & Jebe 2008). These disadvantages might be avoided by the use of allogeneic MSC-products obtained from euthanized or slaughtered horses. Thus, the alternative use of allogeneic MSCs has been proposed for regenerative therapies

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in people (Alison & Caplan 2009; Heng *et al.* 2009) as well as in horses (Watts *et al.* 2011; Iacono *et al.* 2012a; Lange-Consiglio *et al.* 2013). The experimental use of allogeneic equine MSCs for treatment of tendon lesions and other orthopaedic disorders has delivered promising results without significant adverse effects (Carrade & Borjesson 2013; Lange-Consiglio *et al.* 2013). For the *in vitro* experiments, the use of MSCs from euthanized horses would follow the concept of replacement, reduction and refinement in animal experiments. The aim of this study was to compare *in vitro* characteristics of eBM-MSCs obtained from euthanized and live horses particularly focussing on immunophenotype. According to the suggestion of the ISCT (International Society for Cellular Therapy) for the immunophenotyping of human mesenchymal stromal cells, a panel of positive (CD44, CD90, CD105, MHCI) and negative (CD11a/CD18, MHCII) commercially available stem cell markers was assessed which had previously been shown to cross react with equine cells. (Dominici *et al.* 2006; Burk *et al.* 2013). Instead of a commonly used doubling time assay, which takes an exponential growing of the cell population for granted, a newly designed proliferation assay was used to describe the proliferation curve, based on the logistical function that includes lag-, log-, and stationary phase. Additionally, the new proliferation assay enables discrimination between fast-proliferating and slow-proliferating subpopulations within a given cell population, possibly leading to a selection of fast proliferating cells in the future.

Methods

Animals

Animal subjects included in this study were 9 warmblood horses, owned by the Equine Clinic of the University of Veterinary Medicine Hannover, which were first narcotized and later euthanized at the Equine Clinic of the University of Veterinary Medicine Hannover for reasons unrelated to this study. The horses were divided into two groups: In five horses bone marrow aspirates were gained before euthanasia, in five horses bone marrow aspirates were gained after euthanasia; one horse belonged to both groups.

Horses that had undergone previous bone marrow (BM) aspiration were excluded from this study. Particular attention was paid to collect BM from donors from a wide range in age.

Isolation and expansion of BM-MSCs from live horses

Bone marrow was aspirated from the sterna of five horses under general anesthesia (No. 2, 4, 6, 8, 9b according to Table 1) using a 13 G bone marrow biopsy needle (Angiotech, Wyomissing, USA). Horses were sedated with xylazine (Xylazin 2%®, CP-Pharma GmbH, Burgdorf, Germany) and general anesthesia was induced by ketamine (Narketan® 100 mg/mL, Vétoquinol GmbH, Ravensburg, Germany) and midazolam (Midazolam® 5 mg/mL, B.

Table 1. Data of bone marrow donors.

Horse	Bone marrow aspirate	Euthanized/ narcotized	Sex	Breed	Age	Disease
1	1	Eut	Gelding	Warmblood	28 years	Colic
2	2	Nar	Gelding	Warmblood	28 years	Infirmity
3	3	Eut	Mare	Haflinger	23 years	Colic
4	4	Nar	Mare	Warmblood	22 years	Lameness
5	5	Eut	Gelding	Warmblood	12 years	Colic
6	6	Nar	Mare	Warmblood	16 years	Lameness
7	7	Eut	Gelding	Haflinger	7 years	Lameness
8	8	Nar	Mare	Warmblood	3 years	Unknown
9a	9	Eut	Mare	Warmblood	2 weeks premature	Sepsis
9b	10	Nar	Mare	Warmblood	2 weeks premature	Sepsis

Braun Melsungen AG, Melsungen, Germany) using standard drug dosages. The horses were placed in dorsal recumbency and general anesthesia was maintained by isoflurane (Isofluran CP®, CP-Pharma GmbH, Burgdorf, Germany) in clean oxygen.

Horse No. 9a/b (Table 1) was sedated and narcotized as later described for euthanized horses. One sample of bone marrow was harvested under general anesthesia (BM aspirate No. 9, Table 1) and then a second one within 30 min after euthanasia (BM aspirate No. 10, Table 1).

Five millilitre bone marrow aspirates were obtained from sternebra 4 or 5 using ultrasound guidance according to Eydt *et al.* (2014) and carried to the lab within 15 min inside a styrofoam container. The bone marrow samples were centrifuged at 10^3 rcf for 15 min and the generated cell pellets resuspended in 10 mL proliferation medium (pmol/L, Mensing *et al.* 2011). The suspension was filtered through a 70 μm filter and was carefully layered over 14 ml Easycoll® (1.086 g/mL, Biochrom AG, Berlin, Germany). After density gradient centrifugation at 400 rcf for 35 min (without brake) the mononuclear cell population (MNCs) was aspirated and cells were washed two times in PBS (DPBS® 1x, Life Technologies GmbH, Darmstadt, Germany). All cells harvested from 5 mL BM were seeded as passage 0 into two 25 cm² cell culture flasks, containing 5 mL pmol/L. Medium was changed 4–24 h after seeding, and subsequently every 2–3 days. When 70–80% confluence was reached, after 7–22 days, cells were detached using trypsin (0.05% Trypsin-EDTA, Life Technologies GmbH, Darmstadt, Germany) and half of the cells were cryopreserved for later use, the other half was passaged. In the first passage, cells from each aspirate were seeded in two 75 cm² cell culture flasks (1×10^5 to 3×10^5 cells) containing 13 mL pmol/L and medium was changed every 2–3 days until 70–80% confluence was reached and cells were further passaged or cryopreserved. The same procedure was repeated in the passage 2 and 3. Cells in passage 1, 2 or 3 reached confluence after 6–15 days. Approximately 4×10^6 cells were harvested from two 75 cm² flasks and used for flow cytometry analysis. Flow cytometry and proliferation assays were performed with cells from passages 1–3.

Isolation and expansion of BM-MSCs from euthanized horses

Five horses (No. 1, 3, 5, 7, 9a, Table 1) were euthanized for reasons unrelated to this study. The horses were sedated with xylazine (Proxylaz®, Bela-Pharm GmbH & Co. KG, Vechta, Germany) and narcotized using ketamine (Ketamin, 10%®, Bela-Pharm GmbH & Co. KG, Vechta, Germany) and diazepam (Diazepam 10 mg®, Rotexmedica, Trittau, Germany). Subsequently, these horses received T61® (Intervet, Unterschleißheim, Germany) for euthanasia. Standard drug dosages were used for all drugs. Within 30 min after euthanasia, 2×5 mL BM were aspirated and processed as described for the live horses.

Flow cytometry analysis for immunophenotyping

Two positive markers (CD90 and CD105) and one negative marker (MHCII) were selected according to the recommendation of the ISCT for identifying human MSCs [19]. Additionally, the presence of CD44, MHCI (positive markers) and absence of CD11a/CD18 (negative marker) was determined. Cells were incubated with 10% horse serum (donor horse serum, heat inactivated, PAA Laboratories GmbH, Pasching, Austria) in PBS for 15 min at room temperature and washed using washing buffer (WB) containing 98.5% PBS, 1% BSA (PAA Laboratories GmbH, Pasching, Austria), 0.01% NaN_3 (Merck-Schuchardt, Hohenbrunn, Germany) and 0.5% goat serum (Institute for Veterinary Anatomy, Histology and Embryology, Giessen, Germany) to block non-specific antibody binding. Subsequently, cells were pelleted in aliquots containing 2×10^5 cells on a 96-well plate with V-bottom and incubated with primary antibodies (Table 2). Cells were then washed twice with WB and stained with appropriate secondary antibodies (Table 2). Then, cells were washed twice, resuspended in PBS and at least 1×10^5 vital cells were analysed by flow cytometry (Accuri C6®, BD Bisoscience, Heidelberg, Germany) using Accuri C6 software (BD Bisoscience, Heidelberg, Germany). Unlabelled cells, secondary antibody

Table 2. List of antibodies used.

Primary antibody						Secondary antibody				
Name	Isotype	Reactivity	Clone	Dilution	Product number	Fluorescence	Isotype	Reactivity	Dilution	Product number
CD44	Rat IgG2b,k	Mouse	IM7	1:400 (1.25μg/mL)	BD 553131	APC	Goat Ig	Rat IgG	1:600 (0.33μg/ mL)	BD 551019
CD90	Mouse IgG1,k	Human	5E10	1:400 (1.25μg/ mL)	BD 555593	PE	Goat Ig	Mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA	1:800 (0.25μg/ mL)	BD 550589
CD105	Mouse IgG1	Human	SN6	1:500 (2μg/ mL)	AbD Serotec MCA 1557T					
MHCI	Mouse IgG2a	Horse	CVS22	1:200 (5μg/ mL)	AbD Serotec MCA1086GA					
MHCII	Mouse IgG1	Horse	CVS20	1:200 (5μg/ mL)	AbD Serotec MCA1085GA					
CD11a/ CD18	Mouse IgG1	Horse	CVS9	1:200 (5μg/ mL)	AbD Serotec MCA1081GA					
Isotype controle	Corresponding antibodies			Dilution	Product number	Fluorescence	Isotype	Reactivity	Dilution	Product number
Rat IgG2b,k	CD44			1:800 (1.25μg/ mL)	Invitrogen 02-9288	APC	Goat Ig	Rat IgG	1:600 (0.33μg/ mL)	BD 551019
Mouse IgG1	CD105, MHCII, CD11a/CD18			1:200 (5μg/ mL)	Invitrogen 02-6100	PE	Goat Ig	Mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA	1:800 (0.25μg/ mL)	BD 550589
Mouse IgG2a	MHCI			1:200 (5μg/ mL)	Invitrogen 02-6200					

only labelled cells and isotype controls were used as control samples. Two different secondary antibodies without spectral overlap were used for multicolour analyses (Table 2). For negative markers (MHCII, CD11a/CD18) positive controls were performed on eBM derived MNCs, which include hematopoietic stem cells (data not shown).

Gating strategy

Dead cells were marked and excluded from the analysed gate, using the viability dye 7-AAD (BD Bioscience, Heidelberg, Germany) in accordance with the manufacturers′ instruction.

Modified Proliferation assay

The proliferation dye eFluor 670 (eBioscience, Frankfurt, Germany) is equally distributed to daughter cells during mitosis. The manufacturer's staining protocol for suspension cells was adapted to the special needs of adherent equine MSCs.

To synchronize the cell cycle, 3.5×10^6 eBM-MSCs, suspended in DMEM (DMEM 1x, 4.5 g/L D-Glucose, Life Technologies GmbH, Darmstadt, Germany), were placed on a soft shaker at 8°C. After 12 h, cells were washed twice with prewarmed (37°C) PBS and were resuspended in 1.0 ml PBS. Afterwards, cell suspensions were divided into 2×0.5 mL. One portion was thoroughly mixed with 0.5 mL eFluor 670 (1:200). The other portion (control) was mixed with 0.5 mL PBS. Both portions were incubated for 10 min at 37°C in the dark. Staining was stopped by adding 2.5 mL cold pmol/L and by incubation on ice for 5 min (in the dark). Subsequently, the cells were washed three times with pmol/L. Cells were counted by flow cytometry and seeded on 6-well plate at a density of 1×10^3 cells/cm². During a period of 10 days, cells were collected successively at six time points (including lag-, log- and stationary-phase) and were analysed by flow cytometry. Obtained data were analysed by logistic regression using the statistical software program BMDP6D (BMDP/Dynamic, Release 8.1 [Dixon, 1993]). The following parameters were determined and used for comparison among the different donors (Fig. 1):

1. G: boundary value, the maximum cell number attainable from 9.6 cm² culture dish
2. G1: time to reach the half boundary value
3. G2: maximal proliferation speed of the cell population
4. G3: time to reach 1.5×10^5 cells
5. G4: cell number after 150 h of cultivation time

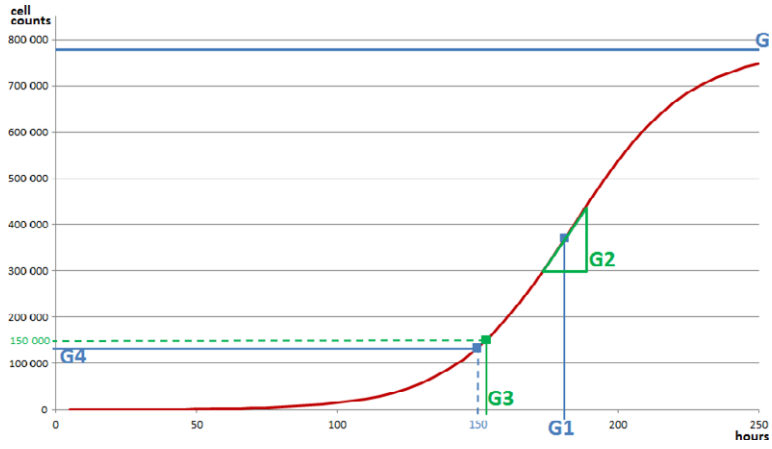

Fig. 1. Example of a proliferation curve of eBM-MSCs. Logistical regression displaying the boundary value, the maximum cell number attainable from 9.6 cm² culture dish (G), the time to reach half boundary value (G1), the maximal proliferation speed of the cell population (G2), the time to reach 1.5×10^5 cells (G3) and the cell number after 150 h of cultivation time (G4).

The calculated values (G–G4) were statistically analysed using a paired-sample *t*-test (eut- and nar-MSCs from similarly aged donors were paired), a covariance analysis and correlation diagrams, in order to analyse differences between the assigned groups of MSCs from narcotized horses and MSCs from euthanized horses and to determine the impact of the donor age. A p-value below 0.05 was regarded as statistically significant.

After 3 days the number of cell divisions undergone by each cell was calculated by measuring the decrease in fluorescence intensity in relation to the initial fluorescence intensity. This information was used to determine the number of cells from different cell generations within a population. The assessment of the generation number was limited to the 6th cell generation as the fluorescence intensity of eFluor 670 decreases after that time. All experiments were performed in triplicate.

Differentiation assays

To confirm tri-lineage differentiation capacity according to the ISCT (Dominici *et al.* 2006), osteogenic, chondrogenic and adipogenic differentiation assays were performed using MSCs obtained from the oldest and the youngest horses as examples of the assigned groups (No. 1, 2, 9a and 9b; Table 1).

Osteogenic differentiation/chondrogenic differentiation

3×10^5 cells were placed into a 15 mL tube within 0.5 mL medium for osteogenic or chondrogenic differentiation and centrifuged for 1 min with 100 rcf. Pellets were cultured under standard conditions, 5% CO_2 at 37°C. Negative controls were supplied with pmol/L instead of differentiation media. Medium was changed every 2–3 days. At day 14, the pellets were fixed in 10% buffered formalin, and processed routinely for histology. Osteogenic differentiation was demonstrated by Von-Kossa staining showing mineralization (Arnhold *et al.* 2007; Cortes *et al.* 2013). Chondrogenic differentiation was demonstrated by Alcian blue staining showing acidic polysaccharides (Colleoni *et al.* 2009; Cortes *et al.* 2013).

Adipogenic differentiation

2×10^4 cells/cm² were seeded on a 24-well plate supplied with 0.5 mL pmol/L. Medium was changed every 2–3 days until 70% confluence. Afterwards, adipogenic differentiation medium was added. Negative controls were formed by supplying pmol/L instead of the differentiation medium. At day 14, the presence of fat droplets was demonstrated using Oil red O staining.

Results

Viable, plastic adherent and proliferating cells were obtained from all 10 bone marrow aspirates.

Immunophenotypic characterisation

All eBM-MSCs from narcotized horses and from euthanized horses presented high percentages (>90%) of CD44 and CD90 positive cells (Figs. 2, 3). The percentages of other positive markers of MSCs (MHCI, CD105) varied widely between MSC populations. MHCI was present in 91.4% ± 7.1 of eut-MSCs and in 76.7% ± 19.1 of nar-MSCs respectively. CD105 was expressed in 33.9% ± 27.2 of eut-MSCs and in 45.7% ± 20.9 of nar-MSCs respectively. None of the detected differences were statistically significant.

The expression of the MSC negative marker (CD11a/CD18) and of MHCII was consistently low (<2.2%) in all nar-MSCs and eut-MSCs.

A covariance analysis showed a significant correlation ($P = 0.016$) between the age of the donor horse and the expression of MHCI. The percentage of MHCI expressing cells increased per year of age by 1%.

Proliferation characteristics

According to the calculated cell divisions, cells were assigned to 4 proliferation types (Figure 4):

1. Type I: Slow-proliferating cells (generation 1 and 2)
2. Type II: Moderate-proliferating cells (generations 3–5)

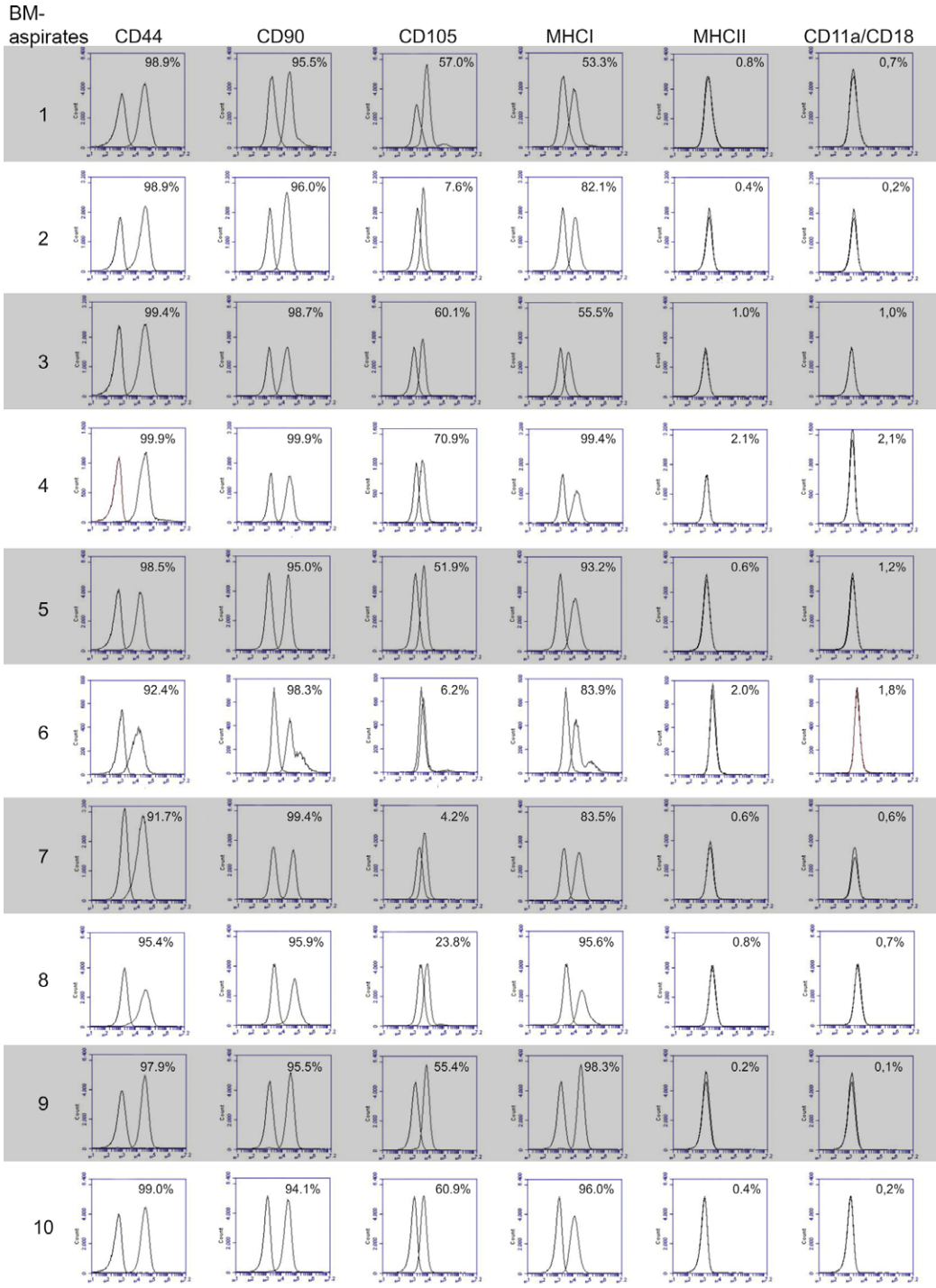

Fig. 2. Flow cytometry analysis. Histograms display fluorescence intensity on *x*-axis and cell counts on *y*-axis. Nar-eBM-MSC and eut-eBM-MSC highly expressed CD44 and CD90. CD105 and MHC I expression varied in wide ranges. CD11a/CD18 and MHCII were expressed by very few cells. Left peaks represent isotype control stainings, right peaks represent antibody staining with corresponding percentage of positive cells (percentages shown as mean of measured doublets).

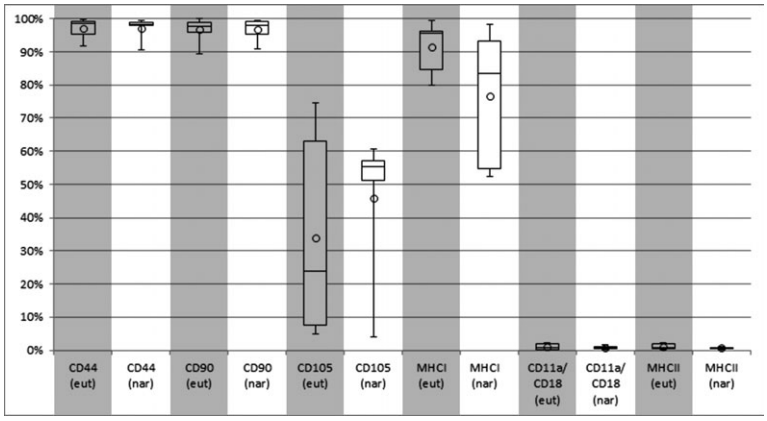

Fig. 3. Marker expression of eBM-MSCs. Box plots of the distribution of percentages of eBM-MSCs from euthanized and narcotized horses, which are positive for the marker expressions. Boxes represent the lower and upper quartiles, lines inside the boxes are medians, whiskers represent minimum and maximum values and circles represent mean values. Eut-MSCs and nar-MSCs show no significant differences ($P > 0.05$).

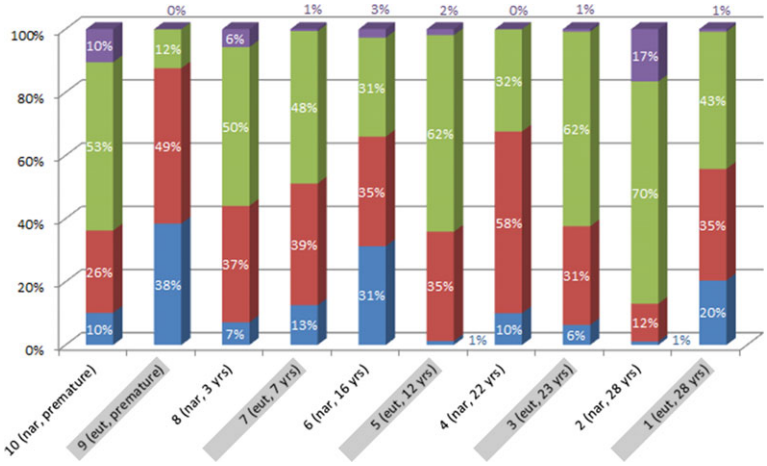

Fig. 4. Proliferation speed of eBM-MSCs. Percentage of slow- (Type I, red), moderate- (Type II, green), fast- (Type III, purple) and non- (Type IV, blue) proliferating cells in expanded cultures from bone marrow aspirates 1–10. Means of triplets displayed.

3. Type III: Fast-proliferating cells (generations >5)
4. Type IV: Non-proliferating cells (generation 0)

From 30 cell cultures (triplets from each culture obtained, that is, 15 nar-MSCs, 15 eut-MSCs), 23 (12 nar-MSC and 11 eut-MSC cultures) contained a heterogeneous mixture of all defined proliferation types, and the remaining 3 nar-MSC and 4 eut-MSC cultures contained no fast-proliferating cells. In 10 nar-MSCs and 11 eut-MSCs, the majority of cells were assigned to the moderate proliferating cell type. In 5 nar-MSCs and in 4 eut-MSCs the majority of cells were slow-proliferating cells. All eut-MSCs contained less than 2.1% fast-proliferating cells. All nar-MSCs (except horse No. 4) contained more than 2.4% fast-proliferating cells (Figure 4).

Correlation diagrams demonstrated that the maximum proliferation speed (i.e. parameter G2) decreased with increasing age of the donor. No statistically significant differences between nar-MSCs and eut-MSCs were detected.

Tri-lineage differentiation

All investigated cells showed their tri-lineage differentiation potential and differentiated into

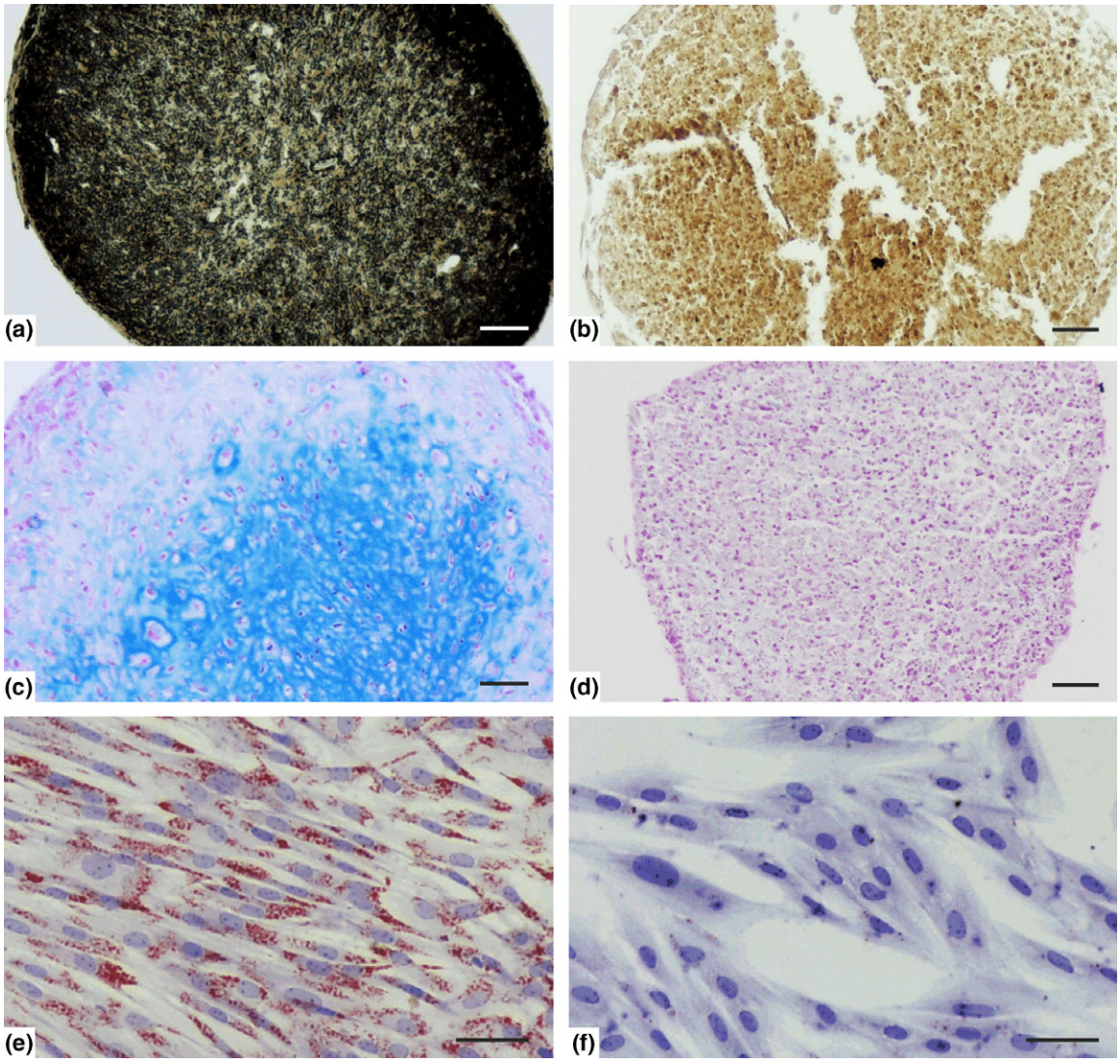

Fig. 5. Results of tri-lineage differentiation assays of eBM-MSCs. Von Kossa staining following osteogenic differentiation (a) and corresponding negative control (b). Alcian blue staining following chondrogenic differentiation (c) and corresponding negative control (d). Oil red O staining following adipogenic differentiation (e) and corresponding negative control (f). Scale bar = 50 μm.

adipogenic, chondrogenic and osteogenic directions (Fig. 5). All controls did not differentiate.

Discussion

The potential use of allogeneic equine MSCs for regenerative therapies could help to overcome disadvantages associated with the harvest of autologous bone marrow. The references show, that autologous bone marrow is usually taken from sedated horses (Brems & Jebe 2008; Bourzac *et al.* 2010), however, there are previous studies, that obtained BM-MSCs also from narcotized horses (Frisbie *et al.* 2009). Although the risks of this method are minimal (Kasashima *et al.* 2011; Eydt *et al.* 2014), the horses are exposed to stress and pain. Another disadvantage is the long post-harvesting time required to produce an injectable autologous cell product (Brems & Jebe 2008). The disadvantages of the autologous sampling method could possibly be avoided if MSCs from one

healthy horse, cryopreserved in many samples, could be used for therapy in many patients. Additionally using MSCs from euthanized horses may increase their availability for research.

Recently, bovine BM-MSCs were successfully harvested from abattoir-derived bovine fetuses (Cortes *et al.* 2013). Vital, proliferating cells, expressing a typical set of MSC markers were obtained and multilineage differentiation was demonstrated. As shown in our data, vital and proliferating cells are also obtainable from euthanized horses. Mesenchymal stromal cells harvest and expansion was successful for at least 30 min after euthanasia, however, the maximum time frame for successful MSC harvest from dead animals remains to be determined, as neither our study nor the study presented by Cortes *et al.* (2013) were designed to investigate this intriguing parameter. In our preliminary experiments, vital eBM-MSCs were successfully obtained even five hours after euthanasia (data not shown).

Unfortunately it was not possible to gain paired samples from each horse before and after euthanasia, as was done for horse No. 9. Therefore, we tried to get samples from horses in a wide range of age in both groups to get an overview about the characteristics of the cells from horses of different ages.

Previous studies suggested the use of fetal abattoir-derived bovine BM-MSCs for regenerative therapies because the cells show typical characteristics of bovine MSCs from live animals (Cortes *et al.* 2013). However, there is no information about possible differences between MSCs obtained from abattoir-derived fetuses and those from live animals.

Although the harvest of BM-MSCs from euthanized horses provides a new opportunity to collect large quantities of cells, potential differences between BM-MSCs obtained from euthanized horses and live horses might restrict the speculative application. Therefore, we performed a comparative evaluation of BM-MSC characteristics that were recognized as crucial parameters for the potential use of MSCs in regenerative therapies. Special attention was paid to use a reliable panel of equine MSC markers to investigate selected criteria relevant for allogeneic applications and to provide detailed information about the particular proliferation capacity of the cells.

Immunophenotyping

The MSC markers used in this study (positive markers CD44, CD90, MHCI, CD105, negative markers CD11a/CD18, MHCII) were selected in accordance with several studies characterizing equine MSC from different sources (Burk *et al.* 2013). Although the utilized antibodies for CD44 and CD90 were produced against non-equine species, they possess a high level of cross reactivity and were previously recognized as reliable markers for equine MSC (Arnhold *et al.* 2007; Mensing *et al.* 2011; Burk *et al.* 2013). To ensure reliable immunophenotyping, we used equine specific antibodies against MHCI, CD11a/CD18 and MHCII. As expected, the vast majority of plastic adherent and expanding cell cultures highly expressed CD44, CD90 and MHCI, but did not show CD11a/CD18 and MHCII reactivity. Although the cross reactivity of anti-human CD105 was confirmed by the manufacturer, the expression of CD105 was highly variable. Therefore, CD105 did not serve as a reliable marker, as previously reported for equine peripheral blood MSCs (Spaas *et al.* 2013).

According to the established marker panel, no significant differences between nar-MSCs and eut-MSCs were present. Thus, cells with the ability to express typical stem cell markers are obtainable from euthanized horses for at least 30 min after euthanasia. This result supports a hypothesis that emphasizes the need for a hypoxic environment in the stem cell niche. It has been proposed that due to a hypoxic environment prevents cellular processes that promote cell differentiation and therefore conserve the undifferentiated status of the stem cell (Ivanovic 2009). It is hypothesized that lack of adverse reactions for allogeneic treatments is due to the absence of MHCII and the immunosuppressive effect of MSCs in general (Heng *et al.* 2009; Rafei *et al.* 2009; Roemeling- Rhijn *et al.* 2013; Li *et al.* 2014). Nevertheless, the range of different conditions the horses had before nar- or eut-MSCs were harvested, did not affect the expression of the markers.

Proliferation capacity

An essential aspect for evaluating MSCs *in vitro* is their capacity to proliferate (Dominici *et al.* 2006).

Moreover, it is assumed that the *in vitro* proliferative capacity is positively correlated with the regenerative capacity of MSCs (Gilbert & Blau 2011). Most assessments of the proliferative capacity are conducted by calculating the cell doubling time (DT) based on the number of seeded cells and the number of cells after a defined time in the culture (Vidal *et al.* 2007; Iacono *et al.* 2012b; Cortes *et al.* 2013). This procedure provides valid results for a phase of exponential cell proliferation. However, proliferation of cell populations *in vitro* is rather characterized by a non-exponential, s-shaped proliferation curve. Thus, the mere doubling time provides only limited information.

In order to detect cells displaying different proliferation capacities within a cell population we conducted a modified proliferation assay utilizing a proliferation dye and flow cytometry. As previously investigated, the data corresponded to the typical s-shaped proliferation curve of expanding cells *in vitro* and therefore allowed detailed analysis of proliferation characteristics. The identification of different cell generations elucidated a heterogeneous mixture of subpopulations with different proliferative capacities. This result was unexpected as other investigated equine MSC cultures were characterized by very homogeneous cell content (Arnhold *et al.* 2007). However, homogeneity of MSCs in these reports was determined according to their fibroblast-like morphological characteristics. Furthermore, the applied proliferation assays were not able to detect differences in proliferation characteristics among cells within a single culture. Pre-selection of fast-proliferating cells could improve the quality of cell products for research and therapy. However, the data obtained here do not provide cellular markers suitable for the predictive selection of those cells.

Although statistical analyses did not reveal any proliferation differences between eut-MSCs and nar-MSCs, there was a negative correlation between the age of the donor horse and the maximum proliferation speed. This might be of importance for selecting appropriate donors. Besides an accelerated *in vitro* expansion speed, eBM-MSCs collected from younger horses might possess advantageous regenerative capacities. This assumption is based on studies that demonstrated telomere shortening in human BM-MSC with increasing donor age, limiting the regenerative capacity of these cells (Baxter *et al.* 2004).

Conclusion

To the best of the authors′ knowledge, the harvest of vital and proliferative eBM-MSCs from euthanized horses has not been previously described. In this study, eBM-MSCs obtained from euthanized horses and from live horses did not differ in terms of expression of MSC markers or proliferative capacities. We suggest that eut-MSCs are suitable at least for scientific studies based on *in vitro* techniques. This would meet the obligation for replacement, reduction and refinement in animal experiments. Moreover, the results obtained here suggest a potential for use of eut-MSC as commercially available products in allogeneic MSC therapies. This assumption has been supported by several clinical studies that successfully utilized allogeneic equine MSCs derived from fetal tissues (Watts *et al.* 2011; Iacono *et al.* 2012a; Lange-Consiglio *et al.* 2013) and from BM of adult donor horses (Guest *et al.* 2008). Moreover, allogeneic MSCs were already successfully applied in clinical human studies (Alison & Caplan 2009; Chang *et al.* 2014; Guest *et al.* 2008).

This study is limited by the number of donor horses and their individual differences in disease, age and genetics. Although these differences might have effects on the characteristics of the harvested MSCs, no significant differences according to the used marker panel were detected. These results justify further investigations to confirm the presented data and to evaluate the possible negative effects of euthanasia on the target cells, with special consideration for the technique and chemicals used for that purpose. The use of MSCs from slaughtered instead of euthanized horses could be considered to avoid influences from diseases or drugs on the obtained cells.

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Conflict of Interest

None of the authors have any financial or non-financial competing interest which could inappropriately influence or bias the content of the paper.

Ethical Statement

Nine warmblood horses were euthanized for reasons other than for this study in the Equine Clinic of the University of Veterinary Medicine Hannover. This study was approved by the Ethics Committee of the University of Veterinary Medicine, Hannover, Foundation, Germany and by the responsible German federal state authority (Lower Saxony State Office for Consumer Protection and Food Safety, 362 33.9-42502-04-11/0572).

Contributions

CSc, CE and CSt have contributed to the conception and design. CSc, LK, FG and CP contributed to collection and compilation of data. CSc, FP, JB and CSt analysed and interpreted the data. CSc and CSt wrote the paper. All authors revised and edited the paper. All authors read and approved the final manuscript.

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