Evaluation of transport conditions for autologous bone marrow-derived mesenchymal stromal cells for therapeutic application in horses (#7537)

First submission

Please read the **Important notes** below, and the **Review guidance** on the next page.
When ready **submit online**. The manuscript starts on page 3.

Important notes

Editor and deadline

María Ángeles Esteban / 17 Dec 2015

Files	6 Figure file(s) 5 Table file(s) Please visit the overview page to **download and review** the files not included in this review pdf.
Declarations	**Involves vertebrate animals.**

Review guidelines

Please in full read before you begin

How to review

When ready **submit your review online**. The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

You can also annotate this **pdf** and upload it as part of your review

To finish, enter your editorial recommendation (accept, revise or reject) and submit.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to **PeerJ standard**, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (See **PeerJ policy**).

EXPERIMENTAL DESIGN

- Original primary research within **Scope of the journal**.
- Research question well defined, relevant & meaningful. It is stated how research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. Negative/inconclusive results accepted. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- Data is robust, statistically sound, & controlled.
- Conclusion well stated, linked to original research question & limited to supporting results.
- Speculation is welcome, but should be identified as such.

The above is the editorial criteria summary. To view in full visit **https://peerj.com/about/editorial-criteria/**

Evaluation of transport conditions for autologous bone marrow-derived mesenchymal stromal cells for therapeutic application in horses

Miguel Espina, Henriette Jülke, Walter Brehm, Iris Ribitsch, Karsten Winter, Uta Delling

Background. Mesenchymal stromal cells (MSCs) are increasingly used for clinical applications in equine patients. For MSC isolation and expansion, a laboratory step is mandatory, after which the cells are sent back to the attending veterinarian. Preserving the biological properties of MSCs during this transport is paramount. The goal of the study was to compare transport-related parameters (transport container, media, temperature, time, cell concentration) that potentially influence characteristics of culture expanded MSCs.

Methods. The study was arranged in three parts comparing I) five different transport containers, II) seven different transport media, four temperatures (4°C vs. room temperature; -20°C vs. -80°C), four time frames (24 h vs. 48 h; 48 h vs. 72 h), and III) three MSC concentrations. Cell viability (Trypan Blue exclusion), proliferation and trilineage differentiation capacity were assessed for each test condition.

Results. No significant differences were found when comparing transport containers, media, temperatures, time frames, or cell concentrations, likely due to the large number of comparisons and small sample size. Highest cell viability was observed using autologous bone marrow supernatant as transport medium, and “transport” at 4°C for 24 h (70.6% vs. control group 75.3%). Viability was unacceptably low with bone marrow supernatant or plasma and DMSO at -20°C or -80°C. Chondrogenic differentiation showed a trend towards being decreased with any transport condition, compared to control cells.

Discussion. In this study, transport conditions were not found to impact viability, proliferation or ability for trilineage differentiation of MSCs, most likely due to the small sample size and large number of comparisons. Future research may be warranted into the possibly negative effect of transport on chondrogenic differentiation.

Evaluation of transport conditions for autologous bone marrow-derived mesenchymal stromal cells for therapeutic application in horses

Miguel Espina[1], Henriette Jülke[2], Walter Brehm[1], Iris Ribitsch[2,3], Karsten Winter[2], Uta Delling[1]

[1] Large Animal Clinic for Surgery, Faculty of Veterinary Medicine, University of Leipzig, Leipzig, Germany

[2] Translational Centre for Regenerative Medicine (TRM), University of Leipzig, Leipzig, Germany

[3] Current address: Equine Hospital, University of Veterinary Medicine, Vienna, Austria

Corresponding author:

Uta Delling[1]

An den Tierkliniken 21, 04103 Leipzig, Germany

Email address: delling@vetmed.uni-leipzig.de

Abstract

Background. Mesenchymal stromal cells (MSCs) are increasingly used for clinical applications in equine patients. For MSC isolation and expansion, a laboratory step is mandatory, after which the cells are sent back to the attending veterinarian. Preserving the biological properties of MSCs during this transport is paramount. The goal of the study was to compare transport-related parameters (transport container, media, temperature, time, cell concentration) that potentially influence characteristics of culture expanded MSCs.

Methods. The study was arranged in three parts comparing I) five different transport containers, II) seven different transport media, four temperatures (4°C vs. room temperature; -20°C vs. -80°C), four time frames (24 h vs. 48 h; 48 h vs. 72 h), and III) three MSC concentrations. Cell viability (Trypan Blue exclusion), proliferation and trilineage differentiation capacity were assessed for each test condition.

Results. No significant differences were found when comparing transport containers, media, temperatures, time frames, or cell concentrations, likely due to the large number of comparisons and small sample size. Highest cell viability was observed using autologous bone marrow supernatant as transport medium, and "transport" at 4°C for 24 h (70.6% vs. control group 75.3%). Viability was unacceptably low with bone marrow supernatant or plasma and DMSO at -20°C or -80°C. Chondrogenic differentiation showed a trend towards being decreased with any transport condition, compared to control cells.

Discussion. In this study, transport conditions were not found to impact viability, proliferation or ability for trilineage differentiation of MSCs, most likely due to the small sample size and large

number of comparisons. Future research may be warranted into the possibly negative effect of transport on chondrogenic differentiation.

Key words: horse, mesenchymal stromal cells (MSCs), transport, viability

Background

Mesenchymal stromal cells (MSCs) are increasingly applied for various diseases in humans (Sharma et al., 2014) and animals (Guercio et al., 2012; Smith, Garvican & Fortier, 2014). In the equine patient, MSCs are predominantly used for treating tendon and ligament injuries (Godwin et al., 2012; Smith et al., 2013). The benefit of MSC injection to treat joint diseases is less well established (Ferries et al., 2014). Mesenchymal stromal cells can be isolated from multiple body tissues including bone marrow (BM), adipose tissue and blood (Sharma et al., 2014; Smith, Garvican & Fortier, 2014). Following isolation, MSCs need to be expanded in culture to provide adequate cell numbers for clinical use. Shipping conditions for transportation of expanded cells to the patient should ensure stem cell viability and maintenance of original cell characteristics, such as purity, identity, differentiation, and proliferation capacity. Various shipping conditions are being used for the transport of expanded MSCs to the patient, but details of validation experiments are either not reported (Godwin et al., 2012), only unfrozen conditions were evaluated (Bronzini et al., 2012; Mercati et al., 2014) or only one frozen condition was evaluated (Garvican et al., 2014). Specifically, Bronzini et al. (2012) used blood-derived MSCs (n=10) and compared 10 different transport media at 4°C, room temperature (RT), and 37°C for up to 72h

using “sterile tubes” of unspecified origin. Mercati et al. (2014) however, used fat-derived MSCs (n=2), assessed one transport media at 4°C vs. RT, and for 24 h and 48 h; the origin of the transport container was not specified either. Finally, Garvican et al. (2014) evaluated BM-derived MSCs (n=3) in 7 different media at 4-8°C and one medium at -78°C for up to 72h, using a single type of specified cryotubes.

When shipping unfrozen cells, it appears that room temperature is superior to 37°C or 4°C when shipping times do not exceed 12 hours (Bronzini et al., 2012). With longer shipping times, keeping the cells at 4°C resulted in higher viability compared to room temperature (Mercati et al., 2014). Further, superior results were obtained using phosphate buffered saline (PBS) compared to culture medium with or without blood serum or fetal bovine serum (FBS) as transport media at temperatures above 0°C up to 12 h (Bronzini et al., 2012). Others report no significant difference in cell viability after 12 h and 24 h in all transport media, but found the most rapid decline in viability over time in suspensions containing biological fluids such as BM aspirate, platelet-rich plasma or serum. Interestingly and in contrast to Bronzini et al. (2012), the least decline was in viability observed with culture medium containing FBS (Garvican et al., 2014). Transport of frozen equine MSCs has been assessed using one condition only (90% allogenic blood serum + 10% dimethyl sulfoxide [DMSO]; -78°C; up to 72h) and resulted in MSC viability of ~80% (Garvican et al., 2014).

Transportation and cryopreservation may impair stem cell functionality and engraftment of human blood-derived haematopoietic stem cells (Lioznov et al., 2008). Also, there have been reports of altered immunosuppressive properties of human MSC following cryopreservation (François et al., 2012).

Another variable to consider is the influence of the transport container, since plastic adherence is a key feature of MSC characteristics. Furthermore, it is known that both surface chemistry and biochemical signals affect MSC proliferation and differentiation (Almodóvar et al., 2010; Wang et al., 2015). Cryotubes are most frequently used in practical settings and in a previous comparable study (Garvican et al., 2014); others did not specify the container under investigation (Bronzini et al., 2012; Mercati et al., 2014;).

Finally, the concentration of cells during transport may impact MSC characteristics. It has been stated that cell concentration may influence biological properties of hematopoietic stem cells (Dlimi, 2012). Also, cell concentration appears to have an impact on cell viability in non-MSC cell lines (De Loecker et al., 1998; Costa et al., 2000).

Therefore, the aim of this present study was to determine the impact of temperature during transport, transport media, transport times, transport containers, and MSC concentrations on equine, bone-marrow derived MSC characteristics.

Our hypotheses were that 1) transport container, 2) transport media, temperature and time, and 3) MSC concentration have an effect on MSC viability, proliferation and trilineage differentiation capacity.

Material and Methods

Study design and general procedures

Study design

105 The study entailed three consecutive parts in which preceding results were applied in the
106 subsequent steps (Fig. 1). In all conditions and controls, cell quality was assessed with respect to
107 viability, proliferation and trilineage differentiation capacity. In part I, recoverable volume for
108 the different containers was determined additionally. The most suitable container was
109 subsequently used throughout the study. In part II, different transport media were tested in
110 positive (four types) and negative (three types) temperatures, each for two time frames (24 and
111 48h, 48 and 72h, respectively). This amounted to 28 different test conditions: 16 at temperatures
112 above 0°C and 12 within the negative temperature range. Finally, in part III, three different MSC
113 concentrations were compared using the best transport media/temperature/time combination as
114 determined in part II. A total of six test conditions were compared; three for the positive and
115 negative temperature, respectively. Individual controls for each condition were defined as the
116 cell characteristics at time point 0, immediately before each test condition was started. Each
117 condition was evaluated using MSCs of six different horses (n=6).
118
119 Sampling of BM and venous blood
120
121 All experiments were approved by the State Animal Care Committee (V12/09, Landesdirektion
122 Leipzig, Free State of Saxony, Germany). Bone marrow was harvested from the sternum of
123 Warmblood foaldings aged 15-16 years as previously described (Delling et al., 2012). Briefly,
124 after intravenous sedation with 0.06 mg/kg romifidine (Sedivet; Boehringer Ingelheim,
125 Ingelheim, Germany) and 0.02 mg/kg butorphanol (Torbugesic; Fort Dodge Veterinär, Würselen,
126 Germany), the area of the sternum was aseptically prepared and locally anesthetized. A BM
127 aspiration needle (Bone Marrow Harvest Needle, Angiotech, Gainesville, USA) was advanced

into the bone and 2 x 20 ml marrow was aspirated into heparinized syringes (10,000 IU heparin-sodium [B.Braun, Melsungen, Germany]/20 ml syringe). Further, 2 x 20 ml venous blood samples were obtained from the jugular vein for the production of autologous plasma using heparinized syringes as described above.

Processing of BM and venous blood

Within 4 h after sampling, the BM was centrifuged in 50 ml conical tubes (Falcon Tube, BD Biosciences, Erembodegem, Belgium; 10 min, 600 x g, 10°C). The bone marrow supernatant (BMS) was collected, filtered (BD Falcon cell strainer, BD Bioscience), and stored at -80°C for further use. From the remaining cellular fraction of the BM, mononuclear cells (MNCs) were isolated by density gradient centrifugation. Briefly, the cellular fraction was diluted with PBS (PAA, Pasching, Austria), layered on 15 ml Ficoll-Paque Premium (GE Healthcare, Uppsala, Sweden) and centrifuged (20 min, 1000 x g, 20°C). The buffy coat containing MNCs was collected, washed twice with PBS and seeded in one T_{75} tissue culture flasks (Greiner bio-one, Frickenhausen, Germany) containing culture medium, i.e. Dulbecco's modified Eagle Medium (DMEM) low-glucose (PAA) supplemented with 10% FBS (Sigma Aldrich, Steinheim, Germany), 1% penicillin/streptomycin (PAA) and 8.9 µg/ml ascorbic acid (Sigma). Cell cultures were kept at 37°C in a humidified atmosphere at 5% CO_2. After 24 h, the adherent cells were washed with PBS and culture medium was added again. For the remaining expansion period, culture medium was changed twice a week until cells reached subconfluency (p0).

In parts I and III of the study, MSCs were harvested, cryopreserved and maintained at -150°C until use. For part II, MSCs of p0 were further expanded until p3 without cryopreservation.

A hemocytometer (Neubauer-improved, Marienfeld-Superior, Lauda-Königshofen, Germany) was used for cell counting throughout the study.
Autologous blood plasma was harvested and stored in an identical manner as the BMS.

MSC viability testing

Cell viability was determined using Trypan Blue stain (Sigma) and the fraction of unstained (viable) cells was determined manually.

Proliferation capacity

To allow comparison of MSC growth characteristics, cumulative doubling rates were calculated for each condition as previously described (Giovannini et al., 2008). For this, cells (p4) were plated at a density of 500 cells/cm^2 on T_{25} tissue culture flasks in culture medium. After seven days in culture, the total number of MSCs per sample was assessed. The process was repeated at weekly intervals until p7. The population doublings were calculated with the equation PD = $\log10 (N/N_0) \times 3.33$, where N is the number of harvested cells and N_0 the number of plated cells. Cumulative population doublings (cPD) for each time point were calculated by adding values of all previous PDs.

Trilineage differentiation potential

For adipogenesis, a modified protocol was used (Giovannini et al., 2008). Briefly, cells (p4) were seeded in duplicate at a density of 5000 cells/cm^2 in 12-well plates (Greiner). At subconfluency, induction medium was added consisting of DMEM/Ham's F12 (PAA), 5% rabbit serum (Sigma), 10% FBS, 1 % penicillin/streptomycin, 1 µM dexamethasone (Sigma), 100 µM indomethacine (Sigma) and 500 µM 3-IBMX (3-isobutyl-1-methylxanthine; Sigma), as well as 1,700 nM insulin (Invitrogen, Karlsruhe, Germany) and maintained for 72 h. Afterwards, cells were fixed with 4% paraformaldehyde (Roth, Karlsruhe, Germany) and the production of intracellular lipid droplets was assessed semi-quantitatively by 0.35 % Oil red O staining (Sigma) counterstained with Mayer hematoxilin (Sigma) and assigning scores of 0-3 for no, low, medium or high numbers of visible fat droplets.

For osteogenesis, cells (p4) were seeded in duplicate at a density of 5000 cells/cm^2 in 12-well plates. At subconfluency, differentiation was induced in DMEM/Ham's F12, 10% FBS, 1% penicillin/streptomycin supplemented with 10 nM dexamethasone, 10 mM ß-glycerophosphate (Sigma) and 0.1 mM ascorbic acid. The medium was changed twice a week. Early and late osteogenesis was evaluated using an alkaline phosphatase stain (Sigma) and Alizarin red stain (Sigma) at day 14 and 21 after induction, respectively. Both stains were assessed semi-quantitatively by assigning scores of 0-3 for no, low, medium and high blue intracellular staining and red stained-extracellular calcium deposition, respectively.

Chondrogenesis was induced in micromass pellet cultures (p4) (Giovannini et al., 2008). To exclude non-viable cells after the simulated transport, all recovered cells were cultured routinely for 12h. Following, only adherent cells were harvested and pellets were prepared as 5 x 10^5 cells in duplicate, placed in 15 ml tubes and centrifuged (5 min, 280 x g, 4°C). Pellets were incubated in chondrogenic medium consisting of DMEM high glucose (4.5 g/l; PAA) supplemented with

196 1% ITS+ Premix (BD Biosciences), 0.1 µM ascorbic acid, 0.4 µM proline (Roth), 100 nM
197 dexamethasone and 10 ng/ml human recombinant TGF-ß$_1$ (BD Biosciences). Medium was
198 replaced twice a week. After 21 days in chondrogenic culture, pellets were fixed for 24 h in 4%
199 paraformaldehyde, dehydrated, embedded in paraffin and cut into 4 µm thick sections. Histologic
200 sections were stained with Safranin O-fast green (Sigma) and Alcian blue (Sigma) to detect
201 extracellular glycosaminoglycan depositions. Masson trichrome staining (Sigma) was performed
202 to evaluate collagen synthesis. All slices were assessed semi-quantitatively using an established
203 scoring system (Bern Score) based on Safranin O-fast green stains and with a maximal score of
204 nine (Grogan et al., 2006). A staining scale from 0 to 3 was used for Alcian blue and Masson
205 trichrome stained sections, respectively.
206 All histologic slides were scored at 20x magnification. Evaluation of the chondrogenic
207 differentiation was blinded to experimental conditions, whereas complete blinding of the
208 adipogenic and osteogenic differentiation was impeded by the consecutive order in the 12-well
209 plates known to the investigator.
210
211 Part I: Evaluation of transport containers
212
213 Cells of p3 were divided into six aliquots. The first aliquot served as control (time point 0) and
214 five aliquots ("transport groups") were transferred to five different transport containers: 1)
215 cryotubes (Art No 126263, Greiner), 2) plastic syringe/plastic tipped plunger (Injekt Innohep, B.
216 Braun), 3) plastic syringe/rubber tipped plunger (Art No 370-003, Henry Schein Vet, Hamburg,
217 Germany), 4) glass syringe/rubber tipped plunger (Art No 663570002, 513010007, and
218 423040001, Gerresheimer, Düsseldorf, Germany), and 5) CellSeal (CellSeal, 2 ml Needleless

Port Foil Covered- Long Tubing Art No 10223-700-47, General Biotechnology, Indianapolis, USA) (Fig. 2). The standardized conditions for the "transport groups" entailed a cell concentration of 10,000,000 MSCs/ml, use of 500µl cell suspension per container, DMEM high glucose + 20% FBS as transport media, and the maintenance for 24 hours at RT (21°C) under light protection. After 24 hours, containers were gently agitated, the contents expelled from the containers into conical tubes and the recoverable volume was measured using a micropipette. Viability, proliferation and differentiation capacity were assessed as described above. Subjective handling characteristics of the individual containers concerning content recovery and ease of use were recorded.

Part II: Evaluation of transport media, temperature and time

The glass syringe was selected for part II and III based on results of part I. Cells of p3 were divided into 29 aliquots: one aliquot served as control and 28 aliquots were used for further testing. Four different transport media, specifically a) PBS, b) autologous BMS, c) autologous blood plasma, and d) HypoThermosol FRS (HypoThermosol FRS, BioLife Solutions, Bothell, USA), were tested at room temperature and 4°C, each for a 24 h and a 48 h "transport" time. Three different transport media, specifically a) CryoStor CS10 (BioLife Solution; which contains 10% DMSO), b) autologous BMS + 10% DMSO (Sigma), and c) autolougous blood plasma + 10% DMSO, were tested at -20°C and [illegible]°C, each for a 48 h and 72 h "transport" time. Each glass syringe contained 500µl cell suspension at a concentration of 10,000,000 MSCs/ml. At the end of each evaluated condition, viability, proliferation and differentiation capacity were assessed as described above.

242

243 Part III: Evaluation of cell concentration

244

245 Conditions with the highest median cell viability in part II at temperatures above and below 0°C,
246 respectively (#5: BMS, 4°C, 24 h; #26: CryoStor, -20°C, 72 h) were further investigated in part
247 III. Specifically, cells of p3 were divided and placed at concentrations of 5 x 10^6, 10 x 10^6, or 20
248 x 10^6 MSCs per ml transport media into glass syringes. As previously, 500µl cell suspension per
249 "transport" container was used and viability, proliferation and differentiation capacity were
250 assessed as described above after 24 h (for temperatures above 0°C) or 72 h (for temperatures
251 below 0°C).

252

253 Data analysis

254

255 Data were tested for normal distribution using the Shapiro-Wilk test. Comparisons between the
256 individual conditions were made using Friedman- and Wilcoxon-Tests. Significance was set at $p < 0.05$
257 <0.05. Post-hoc Bonferroni correction was applied to adjust the p-value. Data were expressed as
258 median and interquartile range (IQR). Statistical analysis was performed with IBM SPSS
259 Statistics (version 22, Armonk, New York, USA).

260

261 **Results**

262

263 Part I: Evaluation of transport containers

264

The recovered volume after "transportation" was highest from the glass syringes (container 4; 91%, 453µl IQR 16µl) and lowest from CellSeal (78%, 390µl IQR 35µl). Plastic syringes 2) and 3) yielded volumes of 88% (440µl IQR 35µl) and 90% (450µl IQR 19µl), respectively. The cryotubes yielded a volume of 83% (415µl IQR 40µl). Differences between the five containers were not significant. Figure 3 depicts the five containers after 24 h of "transport" and immediately before evacuation. Note the cell pellet which formed at the bottom of all containers. Subjectively, we noticed foam formation after the content had been agitated in the cryotube (container 1) and CellSeal (container 5), thus decreasing the recoverable volume. Upon pushing the plunger of the syringes to expel the cell suspension, the glass syringe allowed for smooth, controlled delivery of the cell suspension, whereas both plastic syringes tended to have an irregular resistance during content delivery.

Viability assessed by Trypan Blue staining was lower in all transport groups compared to the control group, i.e. viability at time point 0 (76.8% IQR 14.6%), although this was not statistically significant. There was also no significant difference in viability between the five containers; highest viability was observed in glass syringes (44.0% IQR 20.3%), followed by the plastic syringe/rubber tipped plunger (38.9% IQR 22.9%), the cryotube (38.1% IRQ 31.2%), CellSeal (38.0% IRQ 20.0%), and the plastic syringe/plastic tipped plunger (37.4% IQR 23.9%).

Proliferation capacity, adipogenic, early and late osteogenic differentiation as well as chondrogenic differentiation were not significantly affected by the containers and there were no differences compared to the control group. Results of the chondrogenic differentiation are given in Table 1.

Based on the highest volume of recovery, subjective ease of use, and highest cell viability among the transport groups, the glass syringe (container 4) was chosen as being the most suitable container for the subsequent evaluation.

Part II: Evaluation of transport media, temperature and time

Viability in all test conditions was decreased compared to the control group (Fig. 4, Table 2). Subjectively, at temperatures above 0°C, cells generally had higher viabilities after 24 hours of "transport" compared to 48 hours of "transport". Furthermore, cells kept at 4°C tended to have higher viability than cells kept at RT. With respect to the transport media, we observed highest viabilities with BMS and plasma. None of these observations reached significance due to Bonferroni correction (significance level $p < 0.002$) and the large number of comparisons. The highest viability of 70.6% within the positive range was observed with condition #5 (BMS, 4°C, 24 h).

Within temperatures below 0°C, the highest cell viabilities were found using CryoStor (#25-28), and among these, test condition #26 (CryoStor, -20°C, 72 h) performed best, albeit not statistically significant.

Proliferation capacity, adipogenic, early and late osteogenic differentiation were not affected by any of the "transport" conditions compared to the control group. However, data for conditions #17-24 was not collected due to insufficient cell numbers harvested after the simulated transport.

All tested conditions had lower Bern scores (Safranin O/Fast green stain) compared to the control group, indicating a decreased presence of mature proteoglycans (Fig. 5), but this was not statistically significant. Scores for the Alcian blue stain were similar to those for the Bern scores.

Collagen deposition, evaluated on Masson-Trichrom-stains, was not affected by any of the tested conditions.

Part III: Evaluation of cell concentration

We did not observe significant differences in viability compared to the control group or between any of the two evaluated MSC concentrations after transport, using conditions #5 (BMS, 4°C, 24 h) and #26 (CryoStor, -20°C, 72 h) (Fig. 6), although subjectively, viability following both transport conditions was consistently lower than in the control group.
Cell proliferation and trilineage differentiation capacity were not affected by the test conditions.

Discussion

The current study shows that transport of expanded MSCs can decrease cell viability to levels that would be unacceptable for clinical use. The aim of this study was to determine influence of different transport factors on MSC's viability and ability to differentiate into different cell lineages. We were unable to identify significant differences between conditions tested, possibly due to the small sample number combined with the large number of comparisons between conditions that were performed in this study. Nevertheless, there are several aspects worth discussing.
An effect of transport media on cell survival within the positive temperature range has been reported before (Garvican et al., 2014). In this previous report equine MSC viability declined most rapidly in all allogenic biological fluids compared to culture medium and saline (Garvican

et al., 2014). This is an interesting finding, because even though we did not reach significant results in our study, we observed contrary results: viability was better preserved with autologous BMS or plasma vs. PBS or HyoThermosol. A potential explanation might be complement system activation in allogenic but not autologous combinations resulting in increased MSC lysis. If autologous products are not available, thermal treatment (56°C, 30 min) of allogenic BMS or blood plasma may resolve this problem. However, heat inactivation in turn adversely affects biological products as well (Giard, 1987) and might therefore be controversial. No difference was found in a study assessing equine MSC expansion in allogenenic plasma lysate vs. FBS, i.e. a xenogenic medium (Seo et al., 2013). Unfortunately no comments were made on complement inactivation in either transport media. In contrast to that, no influence on equine MSC survival was found when comparing ten different transport media encompassing PBS, DMEM, with or without the addition of 20% or 80% equine serum (presumably allogenic), or 20% or 80% FBS (Bronzini et al., 2012). Subsequently, PBS was recommended by the authors without further explanation. We used culture medium (DMEM high glucose + 20% FBS) in part I of our study. The observed low viability (37-44%) as well as the inclusion of FBS prompted us to investigate alternative transport media in part II. The addition of FBS to culture medium during transport of equine MSCs was omitted on purpose by others to avoid introducing foreign materials (Mercati et al., 2014). FBS is considered to potentially cause xenogenic immune reactions and may additionally carry the risk of transmitting bovine pathogens such as viruses, bacteria, and prions (Sundin et al., 2007). A lower degree of inflammation was observed after intraarticular injection of autologous MSCs compared to allogenic or xenogenic MSCs, thus reinforcing the importance of potential immunoreaction due to foreign proteins (Pigott et al., 2013).

We observed an unacceptably low viability after transporting MSCs within the negative temperature range using BMS + 10% DMSO or blood plasma + 10% DMSO. This is in contradiction to previous studies where superior viability was obtained by freezing MSCs in 90% allogenic blood serum + 10% DMSO, i.e. viabilities between 60-80% after 48-72 h were observed (Garvican et al., 2014). Differences between protocols may explain contradictory findings, for example the method of thawing: we submerged samples quickly in a 37°C warm water bath. Rapid thawing of mammalian cells is recommended to prevent ice crystal formation and cell lysis, thus, 37°C is the recommended thawing temperature (Katayama et al., 1997; Phelan, 2007).

Another point to discuss is the use of DMSO as cryopreserving agent. DMSO was part of all transport media within the negative temperature range in our study; however, at concentrations used for cryopreservation and temperatures >4°C DMSO is potentially cytotoxic. Adverse and toxic reactions in recipient human patients have been reported (Thirumala, Goebel & Woods, 2013). Finally, DMSO has been described as being capable of inducing differentiation of stem cells into cardiac or neuronal-like cells (Woodbury et al., 2000; Young et al., 2004). These aspects are of concern in the equine patient as well since the entire thawed sample would be injected into the patient by the equine practitioner, including the full amount of DMSO.

Based on our data, CryoStor may be the transport media of choice for shipping frozen equine MSCs. But this conclusion merits caution since to our knowledge no data are available on in vivo compatibility of this product after, for example, intratendinous or intraarticular injection in horses. An alternative concept practiced after transportation of human hematopoietic stem cells, is re-culturing the MSC upon arrival, but this procedure is not feasible for most practicing veterinarians.

The subjectively observed declining viability of unfrozen MSCs over time (24 h vs. 48 h), which was seen in our study in each of the compared test conditions, has been reported consistently before by others (Bronzini et al., 2012; Mercati et al., 2014; Garvican et al., 2014). Interestingly, Bronzini et al. (2012) assessed viability more closely during the first 24 hours, i.e. at 3, 6, 9, 12, and 24 h (and further). They reported a steadily maintained viability up to 12 h; however, a steep decline in viability from 12 h transport time to 24 h was observed. It is also of no surprise that time (in our study 48 h vs. 72 h) appeared to have far less influence on viability of MSCs in a frozen state; this is in accordance with previous findings in equine MSCs (Garvican et al., 2014). While there was no significant difference for temperature (4°C vs. RT) in our study, an advantage of lower temperatures has been previously reported (Mercati et al., 2014). However, a higher rate of equine MSC survival at RT vs. 4°C (and 37°C) was reported by one research group before (Bronzini et al., 2012). There was also no influence of temperature in the negative temperature range (-20 vs. -80°C) in our study. Thus, during transport, it is paramount to maintain temperatures at minimum below -20°C. Temperatures of approximately -80°C are preserved on dry ice but require additional effort and cost during shipment. A temperature of -20°C may or may not be maintained with freezer packs and appropriate insulating packaging. Continuous temperature measurements within the transport box might be necessary for quality control as, to our knowledge, no studies are available concerning this practice. In clinical settings, transport of suspended equine MSCs at 4-10°C was facilitated using freezer packs and insulated boxes (Godwin et al., 2012).

There are discrepancies in MSC viability from previous publications (Bronzini et al., 2012; Mercati et al., 2014; Gravican et al., 2014) compared to our findings. This might be due to the MSC source (blood, adipose tissue, BM), the quantifying technique (manual vs. automated

counting), individual settings within each laboratory, or other unknown factors. Direct comparison of protocols is impeded by curtailed description of protocols in some of the other studies (Bronzini et al., 2012). The slightly decreased viability in our control group (time 0) was most likely due to logistic reasons: the samples for this group were handled together with the test groups, meaning that in part II, 29 samples were assessed at the same time. The resulting prolonged processing time may have influenced viability. This may partially account for the poor survival after freezing MSCs in part II of our study too, because one condition (Cryostor, -20°C, 72h), resulted in part II in 35.5% viability and in part III (where fewer samples were evaluated) in 61.8% viability. Because all samples within each parts of the study were handled equally, comparison between individual settings in each part of the study are still valid in our opinion. This may indicate that, in the clinical setting, cells should be used as soon as possible after they have been thawed.

The undisturbed adipogenic and osteogenic differentiation throughout the study is in accordance with previous findings (Mercati et al., 2014). We found that the chondrogenic differentiation appeared to be affected, though not significantly. Decreased chondrogenic differentiation may be of concern because MSCs are intraarticularly applied in horses for the treatment of osteoarthritis (Ferris et al., 2014) and further investigation into this possible effect is warranted.

Only negligible differences in recovered cell suspension volume were found when the five transport containers were compared. It has to be taken into account, that in part I the containers were tested at RT only; the outcome of the investigation might have been different at other temperatures, e.g. below freezing. The lack in differences between the containers was unexpected, because the material of our chosen transport containers varied considerably.

Counting the recovered cells after the simulated transport would have probably yielded further useful information. Cell count was determined in some (Bronzini et al., 2012; Mercati et al., 2014) but not in other (Garvican et al., 2014) previous studies evaluating the effect of transport on equine MSCs.

We noticed that the liquid content tended to foam after agitation, making aspiration in the cryotube and in the CellSeal more difficult. The foam formation in the glass syringes dissolved faster compared to all other containers. Agitation is necessary because cells tend to sediment quickly to the bottom of the container. We also noticed that even after agitation, cell accumulations sometimes remained visible in the fluid. This was irrespective of the type of container. Protein aggregation and particle formation in prefilled glass syringes is a known effect in the pharmaceutical industry (Jones, Kaufmann & Middaugh, 2005; Gerhardt et al., 2014). Prefilled glass syringes and some plastic syringes contain silicon oil as a lubricant to enable smooth plunger movement. It has been shown that the silicon oil-water and air-water interface combined with agitation are responsible for the aggregating effect (Jones, Kaufmann & Middaugh, 2005). Injecting large protein particles might be of concern in the equine patient, but has not been evaluated specifically. Also, risk of breakage of glass syringes compared to plastic containers has to be considered. In general, the use of syringes as transport containers seems appealing since they are ready-to-use products. However, they are not sterile on the outside and special precaution is required when using the syringes for sterile application. In contrast, a sterile syringe can be used to aspirate the content from cryotubes or CellSeal. We occasionally noticed that aspiration of small volumes from CellSeal was difficult and small pieces of plastic from the seal blocked the 21 G needle used for aspiration. A larger needle might have prevented that, but

444 may result in injection of small plastic pieces into patients' tissues. Additionally, the large port of
445 cryotubes may be a potential entry for contamination during sample aspiration.
446 We compared various cell concentrations during transport, because injecting small volumes and
447 therefore preparation of highly concentrated MSC suspensions might be of interest for the
448 treatment of small tendon defects. In our experience (unpublished obervations) even 1 ml MSC
449 suspension injected into small tendinous lesions (under sonographic guidance) leaked outside the
450 defect and into the peritendinous tissue. This was proven by labeling the MSCs with SPIO (iron
451 oxide particles) and subsequent sequential MRI evaluation. In clinical cases application of 2 ml
452 volume at a concentration of 5 x 10^6 MSCs/ml has been reported (Godwin et al., 2012; Smith et
453 al., 2013). In previous experimental studies assessing MSC transport conditions comparatively
454 lower concentrations of 0.1 x 10^6 MSCs/ml (Bronzini et al., 2012), 1 x 10^6 MSCs/ml (Mercati et
455 al., 2014), and 5 x 10^6 MSCs/ml (Garvican et al., 2014) were evaluated. In our study, no negative
456 impact on MSC viability, proliferation and differentiation capacity was observed even in our
457 highest concentrated solution of 20 x 10^6 MSCs/ml.
458 The high number of comparisons within our study necessitated the application of Bonferroni
459 correction. This fact combined with the observed high variability and low samples numbers are
460 probably the reason for the lack of statistical significance.
461

462 **Conclusion**

463

464 In summary, we could not statistically prove any of our three hypotheses. However, transport
465 media, transport time and the temperature during transport seem to be critical factors potentially
466 influencing MSC quality.

467

468 **References**

469 1. Almodóvar J, Bacon S, Gogolski J, Kisiday JD, Kipper MJ. 2010. Polysaccharide-based
470 polyelectrolyte multilayer surface coatings can enhance mesenchymal stem cell response to
471 adsorbed growth factors. *Biomacromolecules* 11:2629-2639. DOI 10.1021/bm1005799.
472 2. Bronzini I, Patruno M, Iacopetti I, Martinello T. 2012. Influence of temperature, time and
473 different media on mesenchymal stromal cells shipped for clinical application. *Veterinary*
474 *Journal* 194:121-123. DOI 10.1016/j.tvjl.2012.03.010.
475 3. Costa E, Usall J, Teixidó N, Garcia N, Viñas I. 2000. Effect of protective agents, rehydration
476 media and initial cell concentration on viability of Pantoea agglomerans strain CPA-2
477 subjected to freeze-drying. *Journal of Applied Microbiology* 89:793-800.
478 4. Delling U, Lindner K, Ribitsch I, Jülke H, Brehm W. 2012. Comparison of bone marrow
479 aspiration at the sternum and the tuber coxae in middle-aged horses. *Canadian Journal of*
480 *Veterinary Research* 76:52-56.
481 5. De Loecker W, Koptelov VA, Grischenko VI, De Loecker P. 1998. Effects of cell
482 concentration on viability and metabolic activity during cryopreservation. *Cryobiology*
483 37:103-109.
484 6. Dlimi A. 2012. Einfluss der Konzentration unterschiedlicher Zellpopulationen auf die
485 Vitalität kryokonservierter Stammzellkonzentrate [German; Influence of concentration of
486 various cell populations of the viabilty in stem cell preparations] Dissertation Dr.med.,
487 University of Erlangen-Nürnberg, Germany.
488 7. Ferris DJ, Frisbie DD, Kisiday JD, McIlwraith CW, Hague BA, Major MD, Schneider RK,
489 Zubrod CJ, Kawcak CE, Goodrich LR. 2014. Clinical outcome after intra-articular

administration of bone marrow derived mesenchymal stem cells in 33 horses with stifle injury. Veterinary Surgery 43:255-265. DOI 10.1111/j.1532-950X.2014.12100.x.

8. François M, Copland IB, Yuan S, Romieu-Mourez R, Waller EK, Galipeau J. 2012. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-γ licensing. *Cytotherapy* 14:147-152. DOI 10.3109/14653249.2011.623691.
9. Garvican ER, Cree S, Bull L, Smith RKW, Dudhia J. 2014. Viability of equine mesenchymal stem cells during transport and implantation. *Stem Cell Research & Therapy* 5:94. DOI 10.1186/scrt483.
10. Gerhardt A, Mcgraw NR, Schwartz DK, Bee JS, Carpenter JF, Randolph TW. 2014. Protein aggregation and particle formation in prefilled glass syringes. *Journal of Pharmaceutical Sciences* 103:1601-1612. DOI 10.1002/jps.23973.
11. Giard DJ. 1987. Routine heat inactivation of serum reduces its capacity to promote cell attachment. *In Vitro Cellular & Developmental Biology* 23:691-697.
12. Giovannini S, Brehm W, Mainil-Varlet P, Nesic D. 2008. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. *Differentiation* 76:118-129.
13. Godwin EE, Young NJ, Dudhia J, Beamish IC, Smith RKW. 2012. Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. *Equine Veterinary Journal* 44:25-32. DOI 10.1111/j.2042-3306.2011.00363.x.
14. Grogan SP, Barbero A, Winkelmann V, Rieser F, Fitzsimons JS, O'Driscoll, Martin I, Mainil-Varlet P. 2006. Visual histological grading system for the evaluation of in vitro-generated neocartilage. *Tissue Engineering* 12:2141-2149.

513 15. Guercio A, Di Marco P, Casella S, Cannella V, Russotto L, Purpari G, Di Bella S, Piccione
514 G. 2012. Production of canine mesenchymal stem cells from adipose tissue and their
515 application in dogs with chronic osteoarthritis of the humeroradial joints. *Cell Biology*
516 *International* 36:189-194. DOI 10.1042/CBI20110304.
517 16. Jones LS, Kaufmann A, Middaugh CR. 2005. Silicone oil induced aggregation of proteins.
518 *Journal of Pharmaceutical Sciences* 94:918-927.
519 17. Katayama Y, Yano T, Bessho A, Deguchi S, Sunami K, Mahmut N, Shinagawa K, Omoto E,
520 Makino S, Miyamoto T, Mizuno S, Fukuda T, Eto T, Fujisaki T, Ohno Y, Inaba S, Niho Y,
521 Harada M. 1997. The effects of a simplified method for cryopreservation and thawing
522 procedures on peripheral blood stem cells. *Bone Marrow Transplantation* 19: 283-287.
523 18. Lioznov M, Dellbrügger C, Sputtek A, Fehse B, Kröger N, Zander AR. 2008. Transportation
524 and cryopreservation may impair haematopoietic stem cell function and engraftment of
525 allogeneic PBSCs, but not BM. *Bone Marrow Transplantation* 42:121-128. DOI
526 10.1038/bmt.2008.93.
527 19. Mercati F, Pascucci L, Curina G, Scocco P, Tardella FM, Dall'Aglio C, Marini C, Ceccarelli
528 P. 2014. Evaluation of storage conditions on equine adipose tissue-derived multipotent
529 mesenchymal stromal cells. *The Veterinary Journal* 2014; 200:339-342. DOI
530 10.1016/j.tvjl.2014.02.018.
531 20. Phelan MC. 2007. Basic techniques in mammalian cell tissue culture. *Current Protocols in*
532 *Cell Biology*. Chapter 1: Unit 1.1. DOI 10.1002/0471143030.cb0101s36.
533 21. Pigott JH, Ishihara A, Wellman ML, Russell DS, Bertone AL. 2013. Inflammatory effects of
534 autologous, genetically modified autologous, allogeneic, and xenogeneic mesenchymal stem

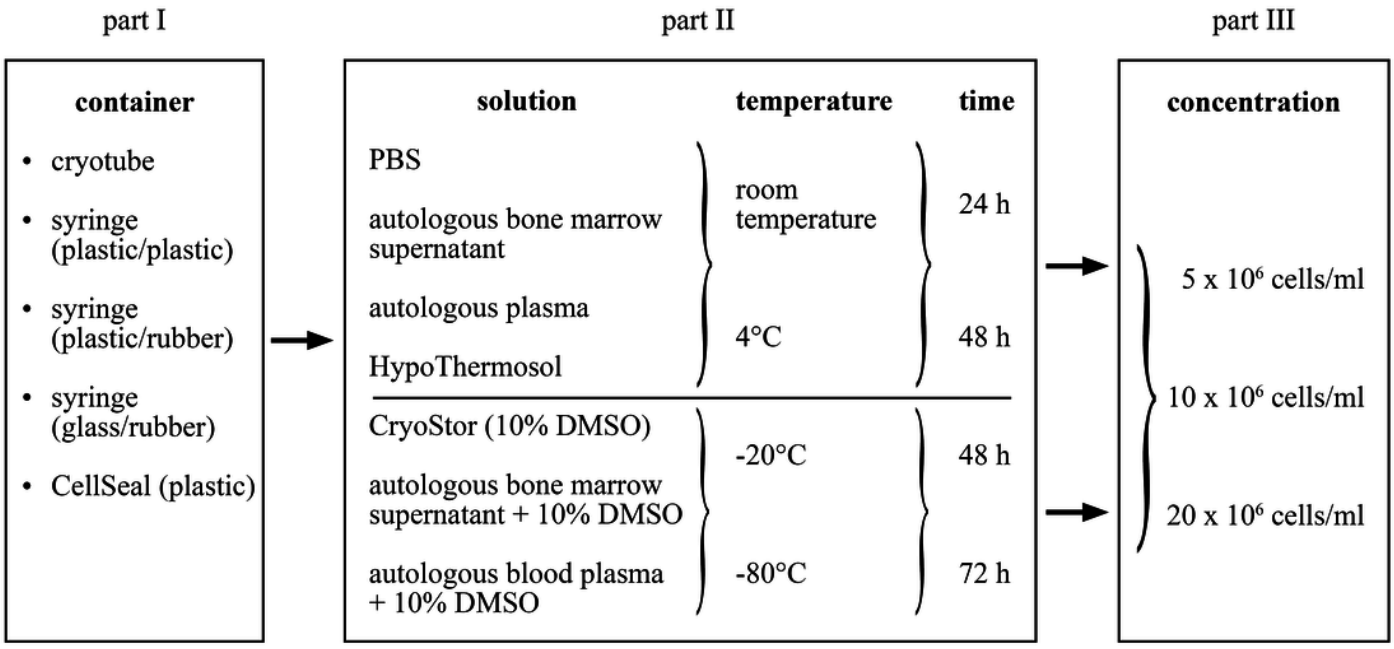
cells after intra-articular injection in horses. *Veterinary and Comparative Orthopaedics and Traumatoogyl* 26:453-460. DOI 10.3415/VCOT-13-01-0008.

22. Seo JP, Tsuzuki N, Haneda S, Yamada K, Furuoka H, Tabata Y, Sasaki N. 2013. Comparison of allogeneic platelet lysate and fetal bovine serum for in vitro expansion of equine bone marrow-derived mesenchymal stem cells. *Research in Veterinary Science* 95:693-698. DOI 10.1016/j.rvsc.2013.04.024.
23. Sharma RR, Pollock K, Hubel A, McKenna D. 2014. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* 54:1418-1437. 10.1111/trf.12421.
24. Smith RK, Garvican ER, Fortier LA. 2014. The current 'state of play' of regenerative medicine in horses: what the horse can tell the human. *Regenerative Medicine* 9:673-685. DOI 10.2217/rme.14.42.
25. Smith RK, Werling NJ, Dakin SG, Alam R, Goodship AE, Dudhia J. 2013. Beneficial effects of autologous bone marrow-derived mesenchymal stem cells in naturally occurring tendinopathy. *PLoS One* 25;8: e75697. DOI 10.1371/journal.pone.0075697.
26. Sundin M, Ringdén O, Sundberg B, Nava S, Götherström C, Le Blanc K. 2007. No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. *Haematologica* 92:1208-1215.
27. Thirumala S, Goebel WS, Woods EJ. 2013. Manufacturing and banking of mesenchymal stem cells. *Expert Opinion on Biological Therapy* 13:673-691. DOI 10.1517/14712598.2013.763925.

557 28. Wang PY, Clements LR, Thissen H, Tsai WB, Voelcker NH. 2015. Screening rat
558 mesenchymal stem cell attachment and differentiation on surface chemistries using plasma
559 polymer gradients. *Acta Biomaterialia* 11:58-67. DOI 10.1016/j.actbio.2014.09.027.
560 29. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow
561 stromal cells differentiate into neurons. *Journal of Neuroscience Research* 61:364-370.
562 30. Young DA, Gavrilov S, Pennington CJ, Nuttall RK, Edwards DR, Kitsis RN, Clark IM.
563 2004. Expression of metalloproteinases and inhibitors in the differentiation of P19CL6 cells
564 into cardiac myocytes. *Biochemical and Biophysical Research Communications* 322:759-
565 765.

1

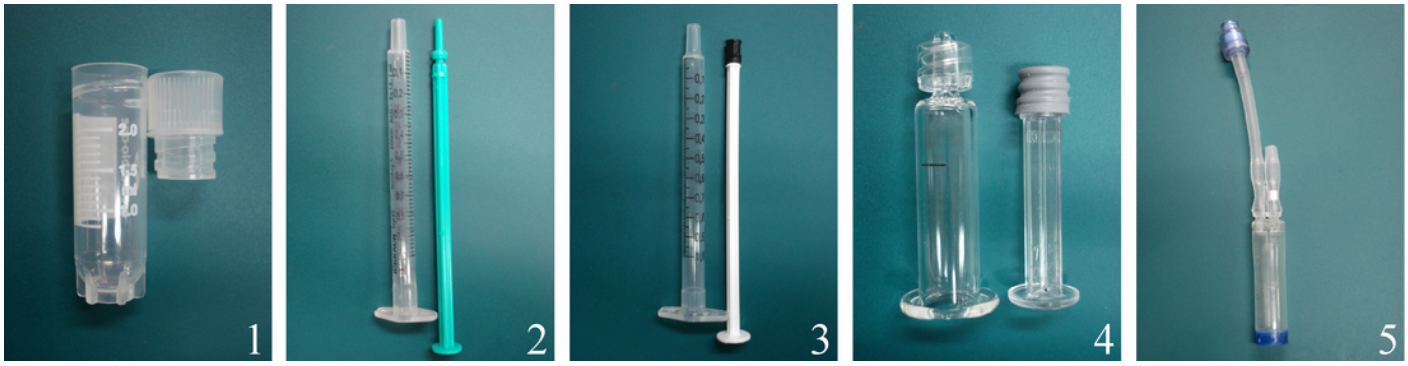
Study design of the three parts of the study



2

Part I: transport containers

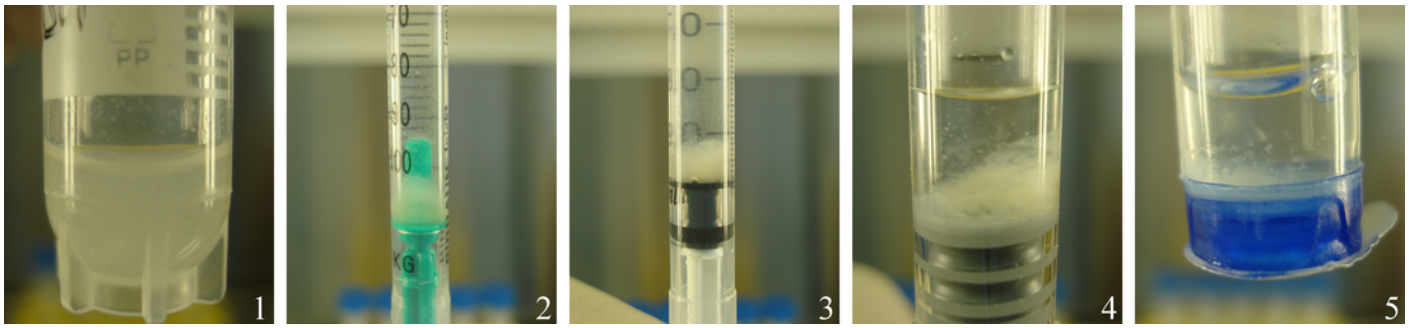
1) cryotube, 2) plastic syringe/plastic tipped plunger, 3) plastic syringe/rubber tipped plunger, 4) glass syringe/rubber tipped plunger, and 5) CellSeal



3

Part I: representative images of the five transportcontainer after 24 h “transportation”and before gentle agitation to dissolve the cell pellet formed at the bottom of the container

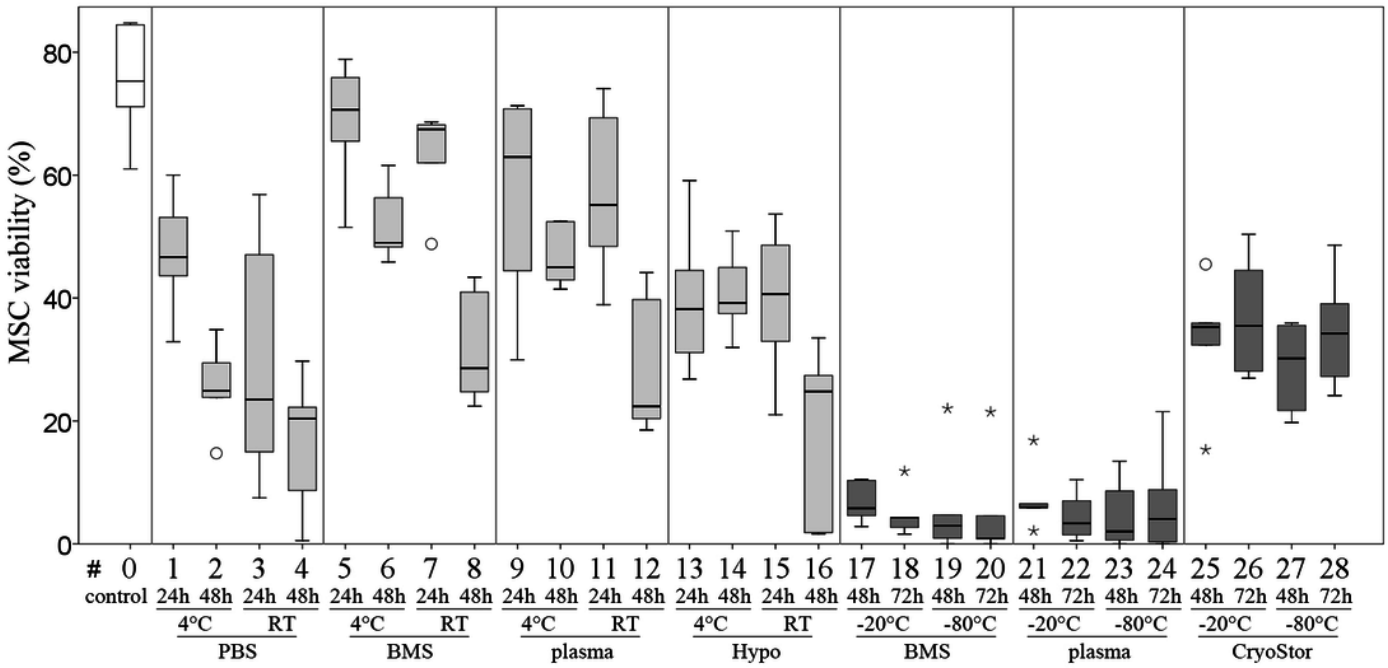
1) cryotube, 2) plastic syringe/plastic tipped plunger, 3) plastic syringe/rubber tipped plunger, 4) glass syringe/rubber tipped plunger, 5) CellSeal



4

Part II: MSC viability

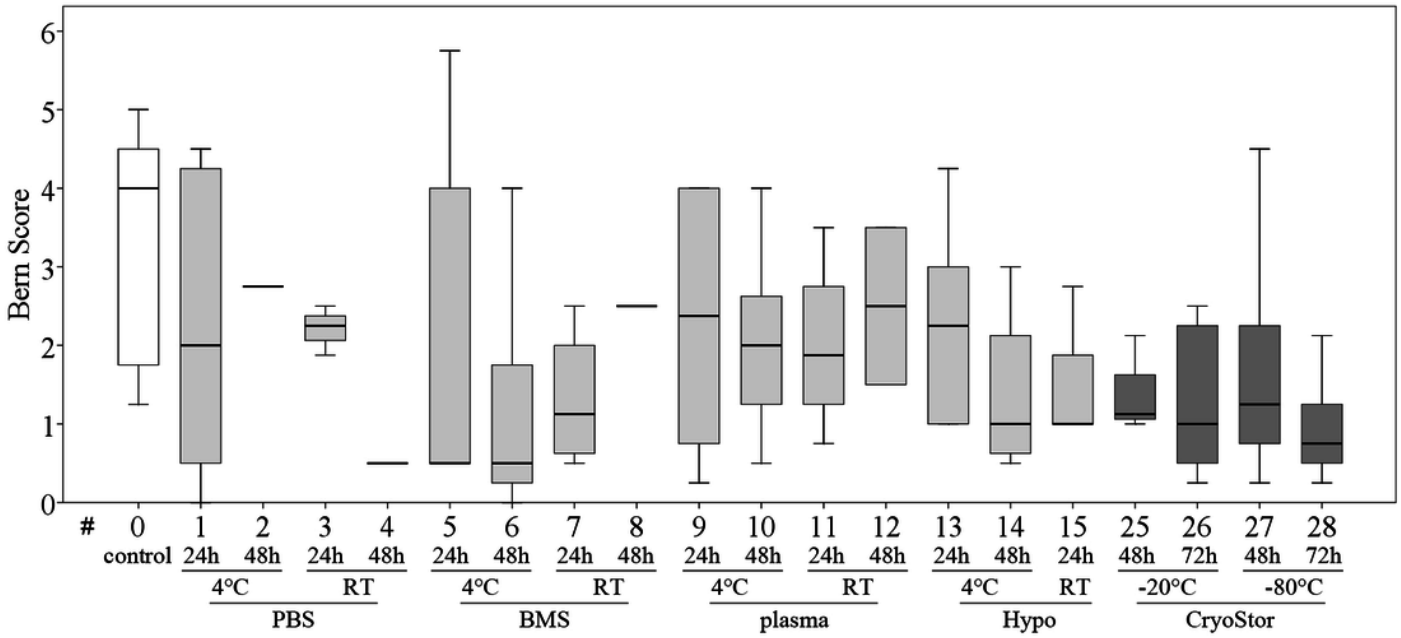
Trypan Blue exclusion (%, median, IQR [box], range [whisker], ° outlier up to 1.5 x box lengths,* extreme values, outside 3 x box lengths) before (#0) and after various “transport” conditions in a positive temperature range (#1-16) and negative temperature range (#17-28); differences did not reach significance level of $p < 0.002$



5

Part II: chondrogenic differentiation capacity

Bern score[23] indicating proteoglycan deposition (median, IQR [box], range [whisker]); a decrease in all test conditions compared to the control group (#0) was noted; differences did not reach significance level of $p < 0.003$; data for #16-24 are missing due to insufficient cell numbers after the “transport”



6

Part III: MSC viability

Trypan Blue exclusion (%, median, IQR [box], range [whisker], ° outlier) under the influence of different cell concentrations before (control) and after “transport”

median	82.6	83.6	81.0	62.0	62.1	63.2	61.8	63.9	63.2
IQR	2.9	4.4	4.8	8.8	7.2	7.3	9.7	6.2	7.0

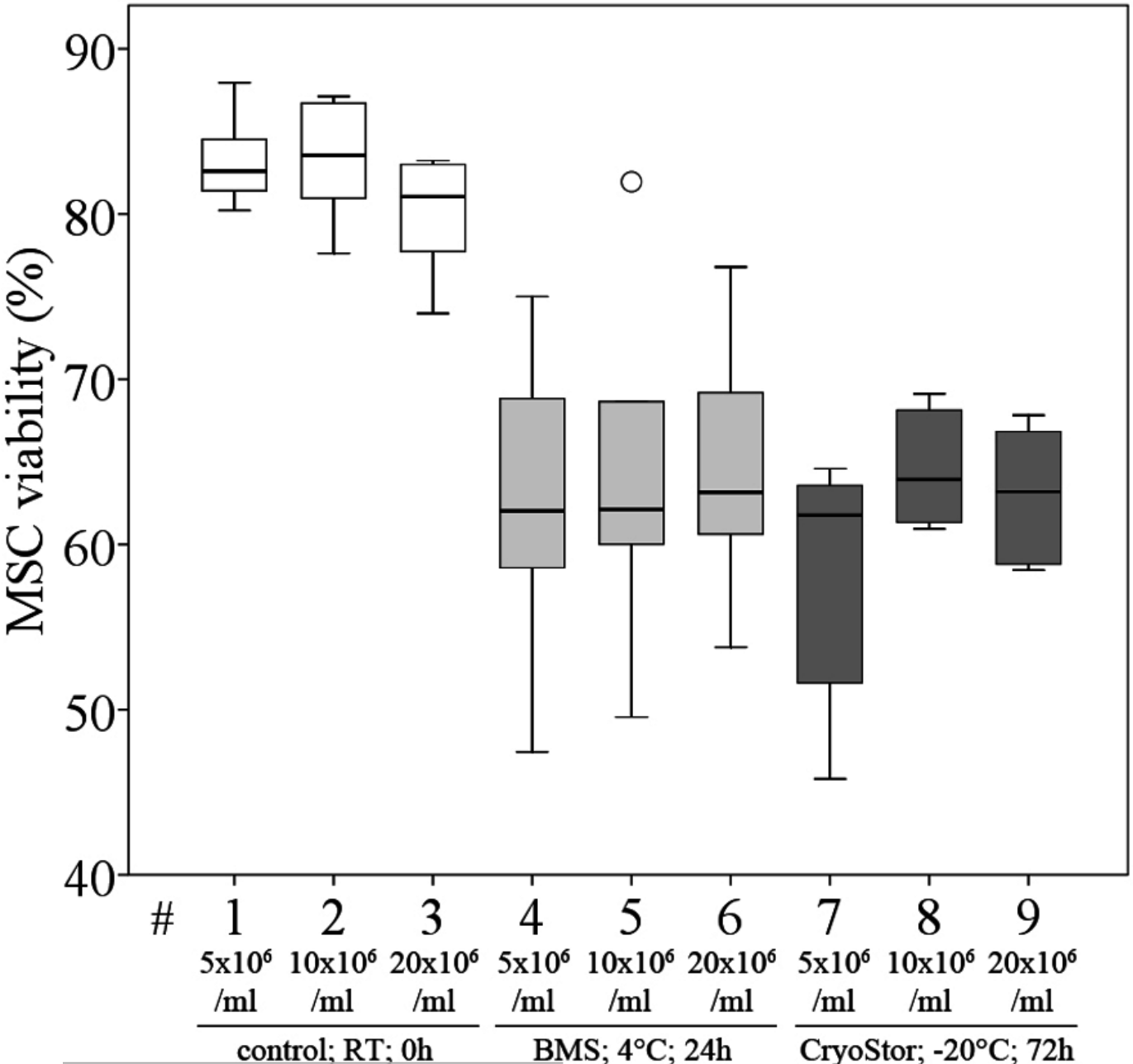


Table 1(on next page)

Part I: chondrogenic differentiation capacity ofMSCs

Bern score[23] (indicating proteoglycan deposition) of MSCs after "transport" using five different transport containers and a control; expressed as median (IQR); container 1) cryotube, 2) plastic syringe/plastic tipped plunger, 3) plastic syringe/rubber tipped plunger, 4) glass syringe/rubber tipped plunger, and 5) CellSeal; there was no significant difference

1

	control	container 1	container 2	container 3	container 4	container 5
Bern score (Safranin-O; 0-9)	2.3 (0.9)	1.3 (4.1)	1.0 (1.1)	1.5 (3.1)	2.3 (2.5)	3.0 (2.8)
Alcian Blue (0-3)	1.8 (0.5)	1.0 (0.8)	1.8 (0.9)	2.0 (0.8)	1.5 (0.4)	1.8 (0.5)
Masson Trichrome (0-3)	2.0 (0)	1.5 (1.0)	1.5 (0.5)	2.0 (0.5)	1.8 (0.5)	1.5 (1.4)

2

Table 2(on next page)

Part II: MSC viability after various “transport”conditions in a positive temperature range and negative temperature range

Viability was assessed using Trypan Blue exclusion; no significant differences ($p < 0.002$)

rank according to viability	transport condition				difference of viability to control (%)	viability (%)	(IQR)
		control	RT	0	n.a.	75.3	(11.0)
1	#5	BMS	4°C	24 h	4.7	70.6	(8.4)
2	#7	BMS	RT	24 h	7.8	67.5	(4.7)
3	#9	blood plasma	4°C	24 h	12.3	63.0	(23.4)
4	#11	blood plasma	RT	24 h	20.1	55.2	(18.7)
5	#6	BMS	4°C	48 h	26.3	49.0	(6.3)
6	#1	PBS	4°C	24 h	28.6	46.7	(9.5)
7	#10	blood plasma	4°C	48 h	30.3	45.0	(8.0)
8	#15	HypoThermosol	RT	24 h	34.7	40.6	(14.7)
9	#14	HypoThermosol	4°C	48 h	36.1	39.2	(6.1)
10	#13	HypoThermosol	4°C	24 h	37.1	38.2	(11.1)
11	#26	CryoStor	-20°C	72 h	39.8	35.5	(16.4)
12	#25	CryoStor	-20°C	48 h	40	35.3	(3.6)
13	#28	CryoStor	-80°C	72 h	41.1	34.2	(11.8)
14	#27	CryoStor	-80°C	48 h	45.1	30.2	(13.8)
15	#8	BMS	RT	48 h	46.7	28.6	(13.2)
16	#2	PBS	4°C	48 h	50.4	24.9	(5.6)
17	#16	HypoThermosol	RT	48 h	50.5	24.8	(19.9)
18	#3	PBS	RT	24 h	51.8	23.5	(32.1)
19	#12	blood plasma	RT	48 h	52.9	22.4	(15.4)
20	#4	PBS	RT	48 h	54.9	20.4	(13.5)
21	#21	blood plasma	-20°C	48 h	69.3	6.0	(0.7)
22	#17	BMS	-20°C	48 h	69.5	5.8	(5.7)
23	#18	BMS	-20°C	72 h	71	4.3	(1.7)
24	#24	blood plasma	-80°C	72 h	71.2	4.1	(8.5)
25	#22	blood plasma	-20°C	72 h	72	3.3	(5.5)
26	#19	BMS	-80°C	48 h	72.4	2.9	(3.8)
27	#23	blood plasma	-80°C	48 h	73.3	2.0	(8.0)
28	#20	BMS	-80°C	72 h	74.4	0.9	(3.8)

1