Aus der Chirurgischen Tierklinik
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Einfluss von Ursprungsquelle und Isolationsmethode auf zellbiologische Charakteristika equiner mesenchymaler Stromazellen

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Meinen Eltern

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Abkürzungsverzeichnis

Dissertationsschrift (Einleitung und Diskussion)

MSC(s)	multipotente mesenchymale Stromazelle(n)
OBS	Oberflächliche Beugesehne
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromid

Publikation 1 (Vergleich der Isolationsmethode im Hinblick auf die Charakteristika von MSCs)

AT	Adipose tissue
AT-MSC	Adipose tissue-derived MSC
BM	Bone marrow
BM-MSC	Bone marrow-derived MSC
Col1A2	Collagen 1A2
di-MSC	MSC isolated by digestion method
DMEM	Dulbecco's modified Eagle medium
ex-MSC	MSC isolated by explant technique
FCS	Foetal calf serum
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GT	Generation time
HBSS	Hank's balanced salt solution
IOD	Index of osteogenic differentiation
IQR	Interquartile range
MNC	Mononuclear cell
MSC	Multipotent mesenchymal stromal cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
P	Passage
PBS	Phosphate-buffered saline
PR	Proliferation rate
qPCR	Real-time quantitative PCR
Scx	Scleraxis
SDFT	Superficial digital flexor tendon
SDFT-MSC	Tendon-derived MSC

UCM	Umbilical cord matrix
UCM-MSC	Umbilical cord matrix-derived MSC

Publikation 2 (Vergleichende Charakterisierung equiner MSCs verschiedener Quellen)

MSC(s)	Multipotent mesenchymal stromal cell(s)
BM	Bone marrow
BM-MSCs	Bone marrow derived MSCs
AdT	Adipose tissue
Ad-MSCs	Adipose derived MSCs
UCB	Umbilical cord blood
UCB-MSCs	Umbilical cord blood derived MSCs
UCT	Umbilical cord tissue
UCT-MSCs	Umbilical cord tissue derived MSCs
TdT	Tendon tissue
Td-MSCs	Tendon derived MSCs
PBS	Phosphate buffered saline
MNC(s)	Mononuclear cell(s)
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
GT	Generation time
PD	Population doubling rate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PR	Proliferation rate
IOD	Index of osteogenic differentation
RT-PCR	Reverse transcriptase-polymerase chain reaction
mRNA	Messenger ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
GAPDH	Glyceraldehyde-3-phosphatase dehydrogenase
BMP-12	Bone morphogenetic protein-12

1 Einleitung

Multipotente mesenchymale Stromazellen (MSCs) sind gefäßassoziierte Bindegewebszellen, die in Körpergeweben als Vorläuferzellen existieren (CAPLAN und BRUDER 2001). In der Literatur werden diese Zellen häufig auch als mesenchymale Stammzellen bezeichnet, wobei der Begriff MSCs aufgrund der Herkunft und Zugehörigkeit der Zellen nach aktuellem Wissensstand exakter ist. Entsprechend der Definition der Internationalen Gesellschaft für Zelltherapie zeichnen sich MSCs durch ihre Fähigkeit zur Plastikadhärenz unter Standardkulturbedingungen, zur multipotenten Differenzierung in vitro und durch ein spezifisches Muster an Oberflächenmarkern aus (DOMINICI et al. 2006; PITTENGER et al. 1999). MSCs entstammen dem Mesoderm, aus welchem während der Embryonalentwicklung Binde- und Stützgewebe sowie glatte Muskulatur und lymphatische Gewebe entstehen (CAPLAN und BRUDER 2001). Aufgrund dieser Verwandtschaft lässt sich die mögliche Differenzierung der MSCs in Zellen des mesodermalen Keimblattes (Adipozyten, Chondroblasten, Osteoblasten) nachvollziehen. Basierend auf dieser Wandlungsfähigkeit der MSCs in vitro erhofft man sich auch eine ähnliche Differenzierungsfähigkeit in vivo. Deshalb sind MSCs immer mehr in den Mittelpunkt von regenerativen Therapieansätzen gerückt, vor allem bei muskuloskelettalen Erkrankungen (CAPLAN und BRUDER 2001; SMITH 2010).

Klinisch eingesetzt werden MSCs unter anderem bereits in der Pferdemedizin zur Behandlung von Sehnenerkrankungen. Solche Pathologien treten häufig bei Sport- und Reitpferden auf und eine Therapie mittels einer intratendinösen Applikation von MSCs erzielt vielversprechende Ergebnisse (AWAD et al. 2003; CROVACE et al. 2010; GODWIN et al. 2012). Zum Beispiel können im Vergleich zur konventionellen Therapie in Form eines kontrollierten Bewegungsprogrammes oder zusätzlicher intraläsionaler Applikation von Hyaluronsäure oder Glykosaminoglykanen nach MSC-Behandlung niedrigere Rezidivraten beobachtet werden (BURK und BREHM 2011; PACINI et al. 2007; RICHARDSON et al. 2007; SMITH 2008), wodurch eine vorzeitige Beendigung der sportlichen Karriere der Pferde und damit wirtschaftliche Verluste seltener auftreten.

Weitere Anwendungsmöglichkeiten von MSCs beim Pferd sind im Bereich von Knorpelerkrankungen in einigen klinischen Fällen beschrieben (MCILWRAITH et al. 2011). Außerdem gibt es erste viel versprechende Untersuchungen oder Studien an Einzelfällen zum Einsatz von MSCs bei Knochenerkrankungen und bei Wundbehandlungen beim Pferd (FRISBIE und SMITH 2010; IACONO et al. 2012; MILNER et al. 2011).

Unabhängig vom Einsatzgebiet der MSCs ist für eine therapeutische Anwendung eine erfolgreiche Isolierung der MSCs erforderlich. Als Quellen stehen dafür viele Gewebe und Körperflüssigkeiten zur Verfügung (YOSHIMURA et al. 2007).

Am häufigsten werden in der Pferdemedizin Knochenmark oder Fett entnommen, um MSCs zu gewinnen (FRISBIE und SMITH 2010; RAABE et al. 2011). Allerdings kann hierbei, abhängig vom Alter des Donortiers, eine verminderte Proliferationsfähigkeit der MSCs beobachtet werden (LOVATI et al. 2011b; TOUPADAKIS et al. 2010). Des Weiteren werden die Gewebe invasiv entnommen. Für Knochenmark erfolgt dazu meist eine Punktion des Sternums, wobei Fehlpunktionen der Thorakalhöhle auftreten können (KASASHIMA et al. 2011). Alternativ steht auch eine Punktion des Hüfthöckers zur Gewinnung von Knochenmark zur Verfügung (DELLING et al. 2012). Beide Entnahmetechniken können im Stehen unter tiefer Sedation durchgeführt werden. Dennoch bleibt ein Risiko bei der invasiven Entnahme durch Abwehrbewegungen des Pferdes bestehen (KASASHIMA et al. 2011). Weniger Komplikationen sind bei der Gewinnung von Fettgewebe zu erwarten. Hierbei kann die Entnahme, ebenfalls am stehenden sedierten Pferd, über eine Hautinzision nahe der Schweifrübe erfolgen. Dadurch ist später eine Narbenbildung möglich, weswegen bei einigen Besitzern aufgrund des kosmetischen Mangels bei ihrem Pferd eine geringere Bereitschaft zur Entnahme von Fettgewebe besteht.

Einfacher erscheint daher die Gewinnung von Geweben wie Amnion und Nabelschnurmaterial, die während der Geburt ohne Komplikationen für Fohlen und Muttertier leicht entnommen werden können (BARTHOLOMEW et al. 2009; HOYNOWSKI et al. 2007). In manchen Studien konnten jedoch nicht immer erfolgreich MSCs aus Nabelschnurblut gewonnen werden (KERN et al. 2006; SCHUH et al. 2009). Weiterhin wurde im Hinblick auf Nabelschnurmaterial von Pferden ein erhöhtes Auftreten an Probenkontaminationen beschrieben (PASSERI et al. 2009). Dies könnte durch die Stallumgebung mit der natürlichen Bakterien- und Schimmelpilzflora verursacht sein, in der die Probenentnahme direkt im Anschluss an das Abfohlen erfolgt.

Außer den Schwierigkeiten einer erfolgreichen und sauberen Isolierungsmöglichkeit weisen MSCs aus geburtsassoziierten Geweben allerdings juvenilere Eigenschaften, wie zum Beispiel die Expression embryonaler Marker, auf (REED und JOHNSON 2008). Dieser damit verbundene vermutete primitivere Charakter der Zellen könnte für einen möglichen universellen klinischen Einsatz dieser MSCs von Vorteil sein.

Im Hinblick auf spezifische therapeutische Einsätze von MSCs könnten sich allerdings auch andere Zellquellen aufgrund spezieller Eigenschaften als geeigneter erweisen. Beispielsweise zeigten MSCs aus Knochenmark eine bessere osteogene Differenzierbarkeit im Vergleich mit MSCs aus Fettgewebe und geburtsassoziierten Geweben (TOUPADAKIS et al. 2010), wodurch sich ein potentieller Vorteil für den Einsatz in der Therapie von Knochenerkrankungen ergeben könnte. Es kann spekuliert werden, dass MSCs aus spezifischen Geweben bereits eine Vordifferenzierung erfahren haben und somit möglicherweise Eigenschaften repräsentieren, die den ursprünglichen Gewebezellen sehr nahe kommen. Deshalb könnten sich für den therapeutischen Einsatz bei Sehnenerkrankungen MSCs aus Sehnengewebe als vorteilhaft erweisen. Erfolgreiche Isolierungen von MSCs aus verschiedenen muskuloskelettalen Geweben sind auch bereits in der Literatur beschrieben (LOVATI et al. 2011a; MENSING et al. 2011). Einschränkend sollte dabei jedoch erwähnt werden, dass nicht alle Gewebe ohne Schäden am Spendertier entnommen werden können und deshalb autologe Anwendungen im Rahmen eines klinischen Einsatzes von MSCs nicht immer realisierbar sind.

Aufgrund der möglichen Unterschiede der MSCs aus den verschiedenen Geweben ist es notwendig grundlegende Aspekte zu den Zelleigenschaften zu erfassen, vor allem im Hinblick auf eine optimale klinische Anwendung von MSCs. Umfassende Studien zum in-vitro-Vergleich von MSCs aus den verschiedenen adulten Geweben sowie den geburtsassoziierten Geweben existierten für equine MSCs bislang nicht.

Unabhängig vom Ursprungsgewebe ist für eine spätere Nutzung von MSCs eine effektive und reproduzierbare Gewinnung der Zellen notwendig. Verschiedenartige Isolierungsmethoden könnten dabei die Qualität der MSCs beeinflussen.

Für die Isolierung von MSCs aus Knochenmark oder Nabelschnurblut ist eine standardmäßige Isolierung mittels Dichtegradientenzentrifugation beschrieben (ARNHOLD et al. 2007; VIDAL et al. 2011). Untersuchungen zu Modifikationen dieser Isolationsmethode für equine Knochenmarks-MSCs zeigten, dass hierbei ein Einfluss auf die gewonnene Zellzahl sowie auf die Expansionsfähigkeit der Zellen beobachtet werden kann (BOURZAC et al. 2010).

Andere Varianten MSCs zu gewinnen bestehen in der Isolierung von Zellen aus soliden Geweben. Dazu sind in der Literatur verschiedene Methoden für die Aufbereitung der Gewebe beschrieben. Klassischerweise wird ein enzymatischer Verdau der Gewebe mittels Kollagenase durchgeführt (RODBELL 1964), der zu einer Freisetzung der enthaltenen

Gewebszellen führt. Diese können dann durch ihre Eigenschaft der Plastikadhärenz weiter in vitro expandiert werden. Auch wenn der enzymatische Gewebeverdau zur Isolierung von MSCs in vielen Laboren weit verbreitet ist, ist nicht eindeutig klar, inwiefern die Zellen in ihren Eigenschaften durch die Wirkung der Enzyme beeinflusst werden (HEFLEY et al. 1981). Studien zum Vergleich von in-vitro-kultivierten Zellen und nativen Zellen im Gewebe zeigten hierbei bereits einen Einfluss der Kultivierung auf die Zellen (GESTA et al. 2003). Eine weitere Beeinflussung der Zelleigenschaften kann auch durch Veränderungen der Kultivierungsbedingungen verursacht werden (MULLER et al. 2011; PARKER et al. 2012), wobei bereits die Umstände einer zweidimensionalen oder dreidimensionalen Kultivierung einen Einfluss zu haben scheinen (TAYLOR et al. 2009). Ähnliche Effekte können dementsprechend auch durch den Kontakt mit der enzymatischen Digestionslösung im Verlauf der Isolationsprozedur vermutet werden. Studien zu Einflüssen des enzymatischen Gewebeverdaus benennen vor allem unspezifisch wirkende Enzyme, vorhandene Toxine in den Enzympräparationen und Gewebezerfallsprodukte als mögliche Noxen, die dann in einer verminderten Proliferation und Expansionsfähigkeit der MSCs resultieren könnten (BURGER 1970; LIU et al. 2009; PATEL et al. 2009). Im Gegensatz dazu könnte auch eine Aktivierung der MSCs durch die Digestionslösung stattfinden, was zu einer erhöhten metabolischen Aktivität der Zellen führen könnte (JAKOB et al. 2003; PADGHAM und PAINE 1993).

Auf der Suche nach alternativen Isolierungsmöglichkeiten von MSCs aus soliden Geweben rückt die Explantationsmethode in den Vordergrund (BAPTISTA et al. 2009; LEE et al. 2011). Hierbei werden Gewebestückchen auf dem Boden adhärenter Zellkulturschalen ausgebracht, wobei die enthaltenen Zellen aufgrund ihrer vorhandenen Migrationsfähigkeit das Gewebe verlassen können und auf dem Boden der Zellkulturschalen anhaften. Eine Vermehrung der plastikadhärenten MSCs erfolgt dann unter Standardkulturbedingungen in einer befeuchteten Atmosphäre bei 37 °C und 5 % CO_2. Zusätzliche externe Noxen sind hierbei während der Isolationsprozedur als minimal einzustufen, da die Zellen direkt aus dem Gewebegerüst auf die adhärenten Böden übergehen können. Umfangreiche Untersuchungen dazu stehen jedoch noch aus. Fraglich bleibt auch, ob nur ein Teil der tatsächlich vorhandenen MSCs, nämlich die randständigen MSCs, mittels des Explantationsverfahrens isoliert werden und somit niedrigere Zellausbeuten erwartet werden können. Erste vergleichende Studien an humanen Zellen liefern gerade im Hinblick auf die erzielbaren Zellzahlen unterschiedliche Ergebnisse (BAPTISTA et al. 2009; LEE et al. 2011). Umfassende Untersuchungen mit besonderem Augenmerk auf Zellcharakteristika und Zellqualität nach verschiedenen Isolationsmethoden existieren dazu bislang noch nicht. Für eine klinische Anwendung von

MSCs sind jedoch ein reproduzierbarer Isolierungserfolg und die Erzielung von hohen Zellzahlen mit guter Zellqualität entscheidend (SEKIYA et al. 2002; YANG et al. 2011). Erkenntnisse über die Einflüsse der Isolationsmethode auf die Zellen sind nicht nur für equine MSCs lückenhaft (BOURZAC et al. 2010), obwohl daraus eine Optimierung ihres Einsatzes in der Pferdemedizin und auch in der Humanmedizin resultieren könnte.

2 Hypothesen

1a. Equine MSCs aus soliden Geweben, die mittels verschiedener Methoden isoliert wurden, unterscheiden sich in vitro in klinisch relevanten Eigenschaften.

1b. Die Isolation von equinen MSCs mittels enzymatischem Verdau führt zu höheren erzielbaren Zellerträgen, beeinflusst jedoch negativ die Wachstumseigenschaften der MSCs.

2. Equine MSCs aus verschieden soliden Geweben und Körperflüssigkeiten weisen unterschiedliche Zellcharakteristika in vitro auf.

3 Ziele

Ziele dieser Studien waren es

1. Equine MSCs aus soliden Geweben mittels enzymatischem Gewebeverdau oder Explantationsverfahren erfolgreich zu isolieren und die gewonnenen Zellen hinsichtlich ihrer grundlegenden Eigenschaften in vitro zu vergleichen.

2. Equine MSCs aus verschiedenen Geweben erfolgreich zu isolieren, die gewonnenen Zellen vergleichend zu charakterisieren und daraus Rückschlüsse auf potentielle Vorteile von MSCs aus bestimmten Geweben für eine klinische Anwendung zu ziehen.

4 Ergebnisse

4.1 Publikation 1 (Vergleich der Isolationsmethode im Hinblick auf die Charakteristika von MSCs)

Isolation of equine multipotent mesenchymal stromal cells by enzymatic tissue digestion or explant technique: comparison of cellular properties

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Isolation of equine multipotent mesenchymal stromal cells by enzymatic tissue digestion or explant technique: comparison of cellular properties

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Abstract

Background

The treatment of tendon lesions with multipotent mesenchymal stromal cells (MSCs) is widely used in equine medicine. Cell sources of MSCs include bone marrow, as well as solid tissues such as adipose tissue. MSCs can be isolated from these solid tissues either by enzymatic digestion or by explant technique. However, the different preparation techniques may potentially influence the properties of the isolated MSCs. Therefore, the aim of this study was to investigate and compare the effects of these two different methods used to isolate MSCs from solid tissues.

Equine adipose tissue, tendon and umbilical cord matrix served as solid tissue sources of MSCs with different stiffness and density. Subsequent to tissue harvest, MSCs were isolated either by enzymatic digestion with collagenase or by explant technique. Cell yield, growth, differentiation potential and tendon marker expression were analysed.

Results

At first passage, the MSC yield was significantly higher in enzymatically digested tissue samples than in explanted tissue samples, despite a shorter period of time in primary culture. Further analysis of cell proliferation, migration and differentiation revealed no significant differences between MSCs isolated by enzymatic digestion and MSCs isolated by explant technique. Interestingly, analysis of gene expression of tendon markers revealed a significantly higher expression level of scleraxis in MSCs isolated by enzymatic digestion.

Conclusions

Both isolation techniques are feasible methods for successful isolation of MSCs from solid tissues, with no major effects on cellular proliferation, migration or differentiation characteristics. However, higher MSC yields were achieved in a shorter period of time by collagenase digestion, which is advantageous for the therapeutic use of MSCs. Moreover, based on the higher level of expression of scleraxis in MSCs isolated by enzymatic digestion, these cells might be a better choice when attempting tendon regeneration.

Keywords

Horse, Regenerative medicine, Collagenase, Cell isolation, Scleraxis

Background

Multipotent mesenchymal stromal cells (MSCs) are described as highly proliferative cells with the capacities of tri-lineage differentiation and plastic adherence [1, 2]. These cells are a promising cell population for alternative treatments of orthopaedic injuries. In equine athletes, MSCs are frequently applied to treat tendon injuries, such as core lesions in the superficial digital flexor tendon (SDFT). Clinical studies have shown more favourable outcomes for this treatment as compared to conventional treatment [3-8].

Currently, the most widely used tissue sources for isolation of MSCs in equine medicine are bone marrow (BM) and subcutaneous adipose tissue (AT) [6, 9, 10].

Although recovery of MSCs from BM is common, there are concerns about the invasive BM aspiration procedure and the potential complications for donor horses [11, 12]. Furthermore, there are cell culture-specific restrictions associated with MSCs derived from BM, such as early cell senescence associated with donor age and limited recovery of MSCs [12-14]. In comparison to BM, various solid tissues, such as AT, tendon tissue or umbilical cord matrix (UCM) appear to yield higher numbers of MSCs that are highly proliferative and that also possess tri-lineage differentiation potential [15-18].

For clinical use, reliably repeatable isolation of an adequate number of MSCs is of great importance [19, 20]. Different protocols are available for the isolation of MSCs from solid tissues [9, 21]. However, the potential impact of the choice of protocol on cell yield and characteristics of equine MSCs has not yet been investigated.

The most frequently used method for isolation of MSCs from solid tissue is digestion by proteolytic enzymes, such as collagenase [22-26]. After digestion, the nucleated cell fraction is released and can be seeded onto plastic culture dishes, where MSCs adhere and thus can be separated from the remaining non-adherent cells.

Other studies have described the isolation of MSCs from solid tissues by a method referred to as the explant technique [27-29]. For this technique, excised tissue is cut into small pieces and plated onto plastic culture dishes. MSCs migrate from the pieces of tissue and adhere to the plastic surface. This method requires less labour and is less invasive to the cells. Moreover, it appears to have less impact on cell viability [28] and might be advantageous due to the initial presence of native tissue and similar physical environment [30].

Enzymatic digestion may negatively affect cellular properties, due to the major alteration of the natural environment of the cells [30], considering that differences in culture conditions also cause alterations of MSC properties [31-33]. However, the impact of differences in the isolation method on MSC characteristics is not yet completely understood [15, 19, 28, 34, 35].

In this study, we isolated MSCs from equine solid tissues by enzymatic digestion or by explant technique. We subsequently compared cell yield, proliferation, migration and differentiation potential of the isolated cells, as well as tendon marker expression, in order to investigate the influence of the isolation technique on characteristics of isolated equine MSCs. For this purpose, we used three types of solid tissues as cell sources for the experiments (AT, SDFT and UCM). All three of these tissues are of different density and stiffness and are known to host MSCs.

Methods

Tissue collection

Equine AT, SDFT and UCM were used as tissue sources for MSC isolation. Subcutaneous AT and SDFT, respectively, were harvested from eight adult horses (mean age: 3.5 years, interquartile range (IQR): 1.75) following euthanasia. UCM samples were collected from 14 foals immediately after birth. Sampling procedures followed the applicable regulations of animal welfare and were approved by the local ethics committee (Landesdirektion Leipzig, A 13/10).

For subcutaneous AT collection, the paraxial caudodorsal gluteal region was clipped and the skin was aseptically prepared. An incision of approximately 10 cm length was made in the skin, and approximately 15 g of subcutaneous AT was obtained with a scalpel and forceps. The tissue was processed immediately.

Tendon samples were obtained from the SDFT of one forelimb of each horse. The palmar region between carpus and fetlock was clipped and the skin was aseptically prepared. After a skin incision of approximately 10 cm length was made, about 15 g of tendon tissue was recovered with a scalpel and forceps and processed immediately.

For UCM collection, approximately 15 cm of the umbilical cord was recovered immediately after foal birth. Umbilical cord tissue was washed with povidone-iodine solution (Braun, Melsungen, Germany) and 70% ethanol (apomix, Halle/Salle, Germany) for disinfection. The umbilical cord was placed in a sterile container with 150 ml phosphate-buffered saline (PBS; PAA, Cölbe, Germany), 0.1% gentamicin (PAA) and 2.5 µg/ml amphotericin B (Life Technologies GmbH, Darmstadt, Germany) and stored overnight at room temperature.

Tissue preparation and cell isolation

Following tissue recovery, the samples were processed under sterile conditions. Blood vessels were dissected from UCM samples prior to further preparation.

Equal amounts, of approximately 6 g, of each specimen were subjected to cell isolation either by tissue digestion or by explant technique.

For digestion, samples of AT, SDFT and UCM were cut into pieces of 0.1-0.2 cm size and washed with Hank's balanced salt solution (HBSS; Life Technologies GmbH). Subsequently, the minced tissue pieces were placed in plastic tubes (BD, Bioscience, Heidelberg, Germany) containing HBSS and collagenase I (Life Technologies GmbH, catalogue number 17100017) and were incubated at 37°C in a continuously shaking water bath. AT was digested for 4 hours in a collagenase I solution at a concentration of 0.8 mg/ml. SDFT was digested for 6 hours at a collagenase concentration of 5.6 mg/ml. UCM was digested for 6 hours at a collagenase concentration of 2.4 mg/ml. After incubation in collagenase solution, remaining tissue pieces were discarded. The digestion solution was filtered with a cell filter (pore size 70 µM; BD Bioscience). The mononuclear cell (MNC) fraction obtained was subjected to two cycles of centrifugation (437 *g*, 5 min, 4°C) and washing in PBS. Subsequently, MNCs were counted using a microscope counting chamber. The cell pellet was resuspended in standard cell culture medium consisting of low glucose (1 g/l) Dulbecco's Modified Eagle Medium (DMEM; Life Technologies GmbH) supplemented with 20% foetal calf serum (FCS; Sigma-Aldrich, Hamburg, Germany, catalogue number F7524), 0.1% gentamicin, and 1% penicillin-streptomycin (PAA). UCM cell culture medium was additionally supplemented with 0.5 µg/ml amphotericin B until first passage to prevent fungal contamination of the cultures [14]. MNCs were seeded onto plastic culture dishes (BD Bioscience) at a density of approximately 20,000 cells/cm^2. Primary cultures (passage [P] 0) were cultivated under standard culture conditions, i.e. humidified atmosphere at 37°C and 5% CO_2, and the culture medium was

changed twice a week. MSCs obtained by digestion, hereafter referred to as "di-MSCs," were passaged by trypsinisation (Trypsin, Life Technologies GmbH) when the cell colonies reached confluency.

For the isolation of MSCs by explant technique, solid tissues were dissected into pieces of approximately 0.5 × 0.5 × 0.5 cm size using a surgical blade and forceps and then washed in PBS. Tissue pieces were placed onto cell culture dishes (TPP, Trasadingen, Switzerland) and covered with standard cell culture medium to allow cell migration from the tissue pieces onto the culture plate (Figure 1). Culture conditions were identical to those following enzymatic digestion. After 7 days, tissue pieces were carefully removed. Primary cultures (P0) were passaged at confluency of colonies to obtain MSCs isolated by explant technique, hereafter referred to as "ex-MSCs."

In the subsequent assays, di-MSCs and ex-MSCs were compared separately for each tissue type to assess the potential effects of the two different isolation techniques. For these assays, seven paired di- and ex-MSC samples derived from adipose and tendon tissue, respectively, were used. Due to a partial contamination of UCM samples, 10 unpaired di- and ex-MSC samples derived from UCM were available for the following assays.

MSC yield

The number of MSCs was counted following trypsinisation at the first cell harvest at confluency of colonies. The yield of MSCs per gram of tissue per primary culture days was calculated according to the following formula:

$$\text{MSC yield} = \frac{\text{cell number at first cell harvest}}{[\text{tissue weight}] \times [\text{number of primary culture days}]}$$

Proliferation assays

From P1 to P7, cells were plated in culture flasks (BD Bioscience) at a density of 3,000 MSCs/cm^2 and incubated to subconfluency in standard cell culture medium under standard culture conditions. Subsequently, MSCs were trypsinised, cell numbers were determined and cells were subjected to seeding as described above. Generation times (GTs) were calculated separately for each passage based on cell counts and culture time according to the following formula:

$$\mathrm{GT} = \frac{\text{cell culture time}}{\text{population doubling}}$$

$$\text{Population doubling} = \frac{\ln\left(\frac{\text{cell number at harvest}}{\text{cell number at plating}}\right)}{\ln 2}$$

Cell proliferation was additionally assessed in P3, as well as in P8, by determining the relative increase in the number of metabolically active cells using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assay, performed according to the manufacturer's instructions. Briefly, 1,000 MSCs per well were seeded onto a 96-well plate and incubated under standard culture conditions. At day 1 CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Mannheim, Germany) was added to the medium and samples were incubated at 37°C, 5% CO_2 for 4 hours. Subsequently, the absorbance at 490 nm was measured using Tecan Safire™ (Magellan™ Software; Tecan Group Ltd., Maennedorf, Switzerland). The same steps were performed in another assay at day 7 after seeding. Proliferation rates (PRs) were calculated using the following formula:

$$\mathrm{PR} = \frac{\text{mean optical density at day 7}}{\text{mean optical density at day 1}}$$

Migration potential

The migration potential of MSCs was determined in P3 by spheroid culture. 5,000 cells per spheroid were cultivated in hanging drops using non-adherent dishes (Greiner Bio-One GmbH, Frickenhausen, Germany) and standard cell culture medium supplemented with methylcellulose (Sigma-Aldrich) (Figure 2a). After 24 hours, spheroids were harvested by rinsing with PBS. The spheroids obtained were plated in standard cell culture medium on adherent 6-well plates (BD Bioscience) and incubated under standard culture conditions to allow the MSCs to migrate out of the spheroids (Figure 2b, c). Photographs (IX51 research microscope; CC-12 digital colour camera; Cell^A software; Olympus Soft Imaging Solutions GmbH, Muenster, Germany) of spheroids and migrating MSCs were taken after 24 hours of incubation (Figure 2d). The migration area was determined following measurement of spheroid size and the area covered by MSCs:

$$\text{migration area} = (\text{area covered by MSCs}) - (\text{spheroid size})$$

In vitro differentiation assays

Adipogenesis assay

1,500 cells/cm^2 in P3 were plated onto adherent 12-well plates (BD Bioscience) in standard cell culture medium to allow cell attachment. After 3 days, adipogenic differentiation was induced by replacement of culture medium with adipogenic differentiation medium consisting of DMEM F-12 (PAA), 15% rabbit serum, 1 µM dexamethasone, 100 µM indomethacin, 500 µM 3-isobutyl-1-methylxanthine, 700 nM bovine insulin (all Sigma-Aldrich) and antibiotics (0.1% gentamicin, 1% penicillin-streptomycin), which had been evaluated previously [36]. After 3 days of incubation, MSCs were fixed with 50% ethanol (Roth, Karlsruhe, Germany) and stored at −20°C until further processing. Cell staining was performed with oil red O solution (Sigma-Aldrich). Two random photographs (IX51 research microscope; CC-12 digital colour camera; Cell^A software) of each well were evaluated by two blinded observers using a scoring system based on the percentage of differentiated cells and the size of intracellular lipid vacuoles (Table 1).

Osteogenesis assay

500 cells/cm^2 in P3 were plated onto adherent 12-well plates in standard cell culture medium. After 3 days of cell attachment, the culture medium was removed and cells were incubated with osteogenic differentiation medium consisting of DMEM F-12, 10% FCS, 0.1 mM L-ascorbate-2-phosphate, 0.1 µM dexamethasone, 10 mM β-glycerophosphate (all Sigma-Aldrich) and antibiotics (0.1% gentamicin, 1% penicillin-streptomycin). Following incubation for 21 and 35 days, cells were fixed with 4% paraformaldehyde (Roth) and stored at −20°C until further processing. For detection of osteogenic differentiation, we used von Kossa staining of extracellular calcium-deposits. Samples with qualitative evidence of differentiation were further assessed as previously described [37]. Briefly, absorbance at 492 nm was determined for stained differentiated samples and undifferentiated controls (Tecan Safire™, Magellan™ Software). Osteogenic differentiation at day 21 and day 35 was quantified by the index of osteogenic differentiation (IOD):

$$\mathrm{IOD} = \frac{\text{optical density of differentiated samples}}{\text{optical density of controls}}$$

Chondrogenesis assay

Chondrogenic differentiation of P3 MSCs was performed in a 3D-pellet culture system. To obtain stable 3D-cell pellets, 500,000 cells per assay were placed into a 15 ml polypropylene centrifuge tube (BD Bioscience) and centrifuged for 5 minutes at 240 *g*. The cell pellets were incubated under standard culture conditions with chondrogenic differentiation medium consisting of high glucose DMEM (4.5 g/l; PAA), 10 ng/ml TGF-β (Acris Antibodies, Herford, Germany), 1% ITS + premix (BD Bioscience), 100 µM L-ascorbate-2-phosphate, 100 nM dexamethasone (both Sigma-Aldrich), 400 nM proline (Roth) and antibiotics (0.1% gentamicin, 1% penicillin-streptomycin). Pellet culture was terminated after 21 days by fixation with 4% paraformaldehyde. Subsequently, the pellets were embedded in paraffin and 6 µm paraffin sections were prepared for Alcian Blue, Masson's Trichrome and Safranin O staining. Pellets showing qualitative evidence of chondrogenic differentiation by Alcian Blue and Masson's Trichrome staining were then semiquantitatively evaluated using the Bern Score [38] based on the Safranin O staining.

Gene expression analysis of tendon markers

Total RNA was isolated from MSC monolayer cultures (P3) using the RNeasy Mini Kit with On-Column DNase digestion (both Qiagen, Hilden, Germany). All steps were performed according to the manufacturer's instructions. RNA was quantified (NanoDrop 1000 Spectrophotometer; NanoDrop Software; Thermo Fisher Scientific, Wilmington, DE, USA) and 1,000 ng of RNA was converted to first strand cDNA with Omniscript Reverse Transcriptase (Qiagen). Fluorescence-based real-time quantitative PCR (qPCR) was performed and monitored using a 7500 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Targeted genes included the tendon markers collagen 1A2 and scleraxis. Each cDNA sample was mixed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Munich, Germany) and gene-specific forward and reverse primers (primer details are shown in Table 2), and the threshold cycle was determined for each sample. Cycling conditions were 40 cycles of denaturation (90°C for 30 sec), annealing (60°C for 30 sec) and elongation (72°C for 30 sec). A set of negative controls was processed in the same manner except that cDNA was replaced with water. The relative copy numbers of target genes were calculated from the standard curve for each gene and normalised to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

The data were processed using PASW Statistics 18 (IBM Deutschland GmbH, Ehningen, Germany). Comparisons were made between di-MSCs and ex-MSCs, for each tissue type separately. The Wilcoxon signed-rank test was used for MSCs derived from AT and from SDFT. Comparison of di-MSCs and ex-MSCs derived from UCM was performed with the Mann–Whitney *U* test. Significance was set at a value of $p \leq 0.05$. Data were reported as median (IQR). Outliers were included in the data analysis. Mild outliers are any data values that lie between 1.5 and 3.0 times and extreme outliers are any data values that lie more than 3.0 times the IQR below the first quartile or above the third quartile.

Results

MSC yield

MSCs were successfully obtained from all three tissues (AT, SDFT and UCM) using both methods, enzymatic digestion and explant technique.

The MSC yield per tissue gram per day, following primary culture was significantly higher using the digestion method in all tissues (Table 3). Furthermore, ex-MSCs required significantly more days in primary culture until the first cell harvest was possible.

Proliferation assays

There were no significant differences in proliferation between di-MSCs and ex-MSCs ($p > 0.05$). However, we did observe trends in proliferation that were dependent on the respective tissue source.

Ex-MSCs derived from AT and SDFT had lower GTs in comparison to the respective di-MSCs (Figure 3A and 3B), indicating faster PRs of ex-MSCs from these tissues. In contrast, ex-MSCs derived from UCM had higher GTs than the respective di-MSCs in most passages (Figure 3C), indicating that in UCM tissue, di-MSCs proliferated faster.

The results from the MTS proliferation assay supported these findings in MSCs from AT and UCM, as higher PRs were found in ex-MSCs from AT and lower PRs in ex-MSCs from UCM, in early as well as late passages (Figure 4A and 4C). However, the finding that ex-

MSCs derived from SDFT proliferated faster than the di-MSCs could not be confirmed by the MTS assay, in which di-MSCs displayed higher PRs (Figure 4B).

Migration potential

Di-MSCs showed a higher migration potential in comparison to the corresponding ex-MSC samples, regardless of the tissue type (Table 4). However, this difference was not significant.

***In vitro* differentiation assays**

Successful induction of adipogenic differentiation was observed in all MSC samples (Figure 5). No distinct differences in the adipogenic differentiation scores were noted between di-MSCs and ex-MSCs, suggesting a similar adipogenic differentiation potential (Table 4).

Extracellular calcium deposits, indicating successful osteogenic differentiation, were observed following von Kossa staining after 21 and 35 days of incubation in all MSC samples derived from AT and SDFT (Figure 6). In di-MSCs and ex-MSCs derived from UCM, one sample in each case did not stain positive for osteogenic differentiation after 21 days of incubation. Following the longer incubation time of 35 days, all UCM-derived ex-MSC samples showed positive von Kossa staining, but one UCM-derived di-MSC sample remained negative. Table 4 provides a summary of IODs of MSCs, measured according to the method of Ostanin et al. (2008) [37]. Interestingly, the IOD for UCM-derived ex-MSCs was lower after 35 days of incubation compared to the IOD at 21 days of incubation.

In terms of chondrogenic differentiation, all ex-MSCs, regardless of the tissue type, were positive for glycosaminoglycans and collagen, as demonstrated by Alcian Blue and Masson's Trichrome staining (Figure 7). Furthermore, all di-MSC samples derived from UCM were able to differentiate towards the chondrogenic lineage. In the case of the di-MSCs derived from AT and SDFT, not all samples showed evidence of chondrogenesis. Two samples from AT and one sample from SDFT did not stain positive for glycosaminoglycans and collagen. Cell pellets that showed successful chondrogenic differentiation, as confirmed by positive Alcian Blue and Masson's Trichrome staining, were further evaluated using the Bern Score [38] following Safranin O staining (Figure 7). No significant differences were observed between di-MSCs and ex-MSCs (Table 4). Di-MSCs and their corresponding ex-MSCs derived from AT and UCM were assigned similar score points. Ex-MSCs derived from SDFT tended to have slightly higher scores compared to their corresponding di-MSCs.

Gene expression analysis of tendon markers

A trend towards higher expression of collagen 1A2 was observed in di-MSCs derived from AT and SDFT, in comparison to the corresponding ex-MSCs. In contrast, di-MSCs derived from UCM displayed a trend towards lower gene expression of collagen 1A2, in comparison to their corresponding ex-MSCs (Figure 8A). Di-MSCs showed higher gene expression levels of the tendon marker scleraxis in comparison to ex-MSCs, regardless of the tissue type (Figure 8B). In MSCs derived from SDFT and UCM, the differences were significant, with p-values of 0.047 and 0.038, respectively.

Discussion

In this study, MSCs were successfully isolated from equine AT, UCM and SDFT by tissue digestion and by explant technique. Further analysis of proliferation, migration and differentiation behaviour of isolated MSCs was performed for comparative evaluation of both MSC isolation methods.

Interestingly, no major differences between cellular properties of di-MSCs and ex-MSCs were observed in the current study, with the exception of a higher level of expression of scleraxis in di-MSCs. However, the tissue digestion method yielded significantly more MSCs in a shorter period of time.

For this study, horses were chosen as donor animals, as the treatment of orthopaedic injuries with MSCs is currently a widely used treatment in equine medicine [3-5]. Furthermore, due to pathophysiological similarities between human and equine orthopaedic diseases, the horse is an appropriate model for orthopaedic research in human medicine [39].

Three different solid tissues, AT, SDFT and UCM, were chosen for exemplary evaluation of MSC isolation methods. These tissues were considered suitable as they were already known to host MSCs [15, 16, 18] and also because their different densities and stiffness allowed for evaluation of protocols under different conditions.

Different techniques exist for isolation of MSCs from diverse sources. In this study, both a standard tissue digestion using collagenase and MSC isolation by explant technique were performed. Enzymatic digestion by collagenase, first described by Rodbell (1964) [40], is a

widely used method for degradation of the collagen network of solid tissue. Nonetheless, some studies have described disadvantages to this method, such as the relatively high costs of reagents, time-consuming labour and inconsistent results due to heterogeneous preparations of pure collagenase solutions [41-44]. To avoid the latter, a uniform batch of collagenase has been used in this study.

Previous studies have investigated other enzymatic methods for MSC isolation, such as the use of liberase, trypsin and hyaluronidase, in order to achieve a reproducible and qualitatively improved tissue digestion and avoid damage to the isolated cells, as an alternative to crude collagenase digestion [20, 41, 45, 46]. However, the use of these alternative enzymatic methods is not without controversy [47]. Therefore, in the present study, standard enzymatic digestion using collagenase was performed for the comparison of MSC isolation techniques. In order to achieve the mildest enzymatic treatment, collagenase concentrations and incubation times were adapted to the requirements of the tissues used as MSC sources, which had been evaluated earlier (unpublished data), as previously suggested by others [30, 47-49].

Several studies have reported the effects of enzymes, endotoxin and chemotactic tissue breakdown products on the phenotype and behaviour of cells [30, 41, 47, 50, 51]. Therefore, we considered a non-enzymatic isolation technique to recover MSCs, which would potentially be less affected and damaged than by an enzymatic isolation technique [28, 52, 53]. In this study, the isolation of MSCs by explant technique was performed and compared to collagenase digestion. The obvious benefit of this non-enzymatic cell isolation technique is that the procedure is simpler in comparison to the enzymatic method and does not require expensive enzymes.

We hypothesized that collagenase digestion affects the isolated cells, while the explant technique does not, thus resulting in differences between the cellular properties of di-MSCs and ex-MSCs. However, this hypothesis was not supported by the present study, as no major differences were found regarding most cellular properties investigated here.

The most important difference between the two MSC isolation techniques was that collagenase digestion yielded significantly more MSCs than the explant technique, which is consistent with published data [28, 44, 47]. A possible explanation might be that only cells located at the tissue margin can migrate out of the tissue in the explant technique, so that not all MSCs residing in the tissue can be collected when using this technique. A practical option

to improve the MSC yield from explant cultures might be to minimize the size of the tissue samples. The MSC numbers per gram of tissue that were obtained by each isolation method in this study are within a similar range to MSC yields reported in other studies [21, 28]. Despite significant differences between cell yields, which might suggest differences between the isolated cell populations, di-MSCs and ex-MSCs displayed similar characteristics during further analyses.

Variation in isolation protocols, as well as alteration of culture conditions, have been reported to influence proliferation of MSCs [31, 32, 34]. This could be due to the fact that some extrinsic substances may be toxic and induce cell death [31, 32] and, therefore, cause variation in viability and expansion potential. In the present study, di-MSCs required less time for primary cultivation in comparison to ex-MSCs. It is likely that this effect was due to the fact that digestion initially makes more cells accessible, rather than to differences in proliferation potential of di-MSCs. In all subsequent passages, when the initial seeding density was standardised, proliferation of di-MSCs and ex-MSCs was similar.

Migration potential of MSCs is considered important for their integration into the host tissue during therapeutic applications. Several studies have reported inhibition or increase of migration capacity by different drugs *in vitro* [31, 54, 55]. Furthermore, a comparative study of different protocols for isolation of BM-MSCs also showed that isolation conditions affect migration ability [34]. Similarly, the technique used to isolate MSCs may affect the ability of MSCs to migrate from solid tissues. In the present study, di-MSCs derived from all the tissues studied, showed a trend towards increased migration activity in comparison to ex-MSCs. This finding was surprising, given that the ex-MSCs had initially been selected based on their migratory capacity during the isolation procedure. Further studies to investigate these effects are necessary. Furthermore, the cultivation of MSCs in a 3D-spheroid assay may be advantageous for injection of MSCs. The investigation of migration potential in these spheroids could potentially be used to assess the ability of the applied MSCs to leave the spheroids and migrate into surrounding host tissue.

In this study, no significant differences were observed in the adipogenic, osteogenic or chondrogenic differentiation capacity between di-MSCs and ex-MSCs. This finding is consistent with results of previous studies in which different methods for isolation of MSCs were compared [28, 34]. In contrast, several studies have demonstrated that changes in culture conditions or cultivation alone seem to affect cellular properties such as the differentiation

potential [31, 51, 56]. Due to standardised and optimised differentiation conditions, such influences on differentiation potential were avoided in the present study.

Analyses of osteogenic and chondrogenic differentiation were performed using standard protocols described in the literature [57]. The observed decrease in IOD for UCM-derived ex-MSCs following longer incubation could be due to partial cell detachment during the longer cultivation time.

The adipogenic differentiation protocol was modified based on an evaluation of different protocols for equine MSCs [36], as insufficient adipogenic differentiation of equine MSCs has been repeatedly reported after standard induction [26, 58]. The modification of the protocol included supplementation of adipogenic differentiation medium with rabbit serum, of which the benefit for adipogenic differentiation has been previously described [26, 36, 58].

Significantly higher expression levels of the tendon phenotype-related gene scleraxis [59-62], were found in MSCs isolated by enzymatic digestion. Alterations in expression levels of other target genes following the digestion technique have been shown in several studies [51, 56, 63]. Potentially, these alterations are caused by the altered environmental conditions the cells are subjected to during enzymatic tissue digestion. It could be also hypothesised that collagen breakdown products from the digested tissues trigger upregulation of the scleraxis gene. There were no noticeable differences in expression levels of collagen 1A2, one of the key components of tendon matrix [64, 65], between di-MSCs and ex-MSCs. It is possible that collagen expression is more stable to influence by extrinsic factors. This hypothesis is supported by published data showing that no variations in transcription level of collagen were observed following supplementation of cell culture medium with ibuprofen [33]. However, only the expression of collagen 1A2 was investigated in present study. During tendon degeneration, initially there is an increased level of collagen 3 fibers which are probably only later replaced by collagen 1 fibers [65]. Hypothesising that collagen breakdown products produced during tissue digestion simulate the early phase of healing and thus stimulate upregulation of tendon markers, collagen 3 expression might be upregulated rather than collagen 1 expression. Still, whether a higher expression of tendon markers *in vitro* reflects the situation *in vivo* and is a reliable indicator of the effect of MSCs on tendon healing remains to be evaluated.

The cell population harvested by enzymatic digestion is potentially heterogeneous, and this raises the question as to whether the isolated cells are in fact MSCs. Further evaluation of cellular properties is important to determine whether these cells represent tissue specific cells, such as tenocytes, or display characteristics of MSCs [33, 59, 64].

Cells isolated in this study were identified as MSCs based on their capacities for self-renewal, plastic-adherence and tri-lineage differentiation. These characteristics are regarded as minimal, but adequate, criteria for identification of MSCs [26, 34, 66].

Evaluation of expression of specific stem markers would have provided more accurate information. However, in contrast to human cells, an established set of equine MSC-specific cell surface markers is not yet available due to the limited reactivity of available antibodies with equine epitopes [67, 68].

Despite the lack of evaluation of MSC markers, the results of this study show that the isolation method has no major influence on cellular growth and tri-lineage differentiation characteristics. Furthermore, no negative effects of collagenase digestion on the isolated cells were observed. Our results are in accordance with the suggestion that alterations in experimental conditions are of minor importance to cell behaviour in comparison to cell source and interdonor variability [20]. Nevertheless, optimisation and standardisation of isolation protocols are required in order to improve comparability of results obtained in different studies [21, 35].

Conclusions

Collagenase digestion and the explant method are both feasible and effective techniques for isolation of MSCs from solid tissues. In this study, the MSCs obtained via both methods displayed similar growth characteristics and tri-lineage differentiation capacities. However, isolation of MSCs from solid tissues by digestion appears advantageous for therapeutic use due to the higher obtainable MSC yields with less time in primary culture. Furthermore, the higher gene expression levels of scleraxis in di-MSCs suggest a potentially more effective role for these cells in tendon regeneration. Further investigation to confirm this hypothesis is required.

Abbreviations

AT, Adipose tissue; AT-MSC, Adipose tissue-derived MSC; BM, Bone marrow; BM-MSC, Bone marrow-derived MSC; Col1A2, Collagen 1A2; di-MSC, MSC isolated by digestion method; DMEM, Dulbecco's Modified Eagle Medium; ex-MSC, MSC isolated by explant technique; FCS, Foetal calf serum; GADPH, Glyceraldehyde 3-phosphate dehydrogenase; GT, Generation time; HBSS, Hank's balanced salt solution; IOD, Index of osteogenic differentiation; IQR, Interquartile range; MNC, Mononuclear cell; MSC, Multipotent mesenchymal stromal cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; P, passage; PBS, Phosphate-buffered saline; PR, Proliferation rate; qPCR, Real-time quantitative PCR; Scx, Scleraxis; SDFT, Superficial digital flexor tendon; SDFT-MSC, Tendon-derived MSC; UCM, Umbilical cord matrix; UCM-MSC, Umbilical cord matrix-derived MSC

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CG designed the study, collected and processed the specimens, assembled and analysed the data and drafted the manuscript. WB supervised the study and helped with editing and revision of the manuscript. JB participated in the design of the study, sample collection and processing, contributed to data analysis and interpretation and helped with editing and revision of the manuscript. HJ contributed to the design of the study and data interpretation. CS provided technical and scientific advice. IR helped in the design of the study, sample collection and processing, contributed to data interpretation and helped with editing and revision of the manuscript. All authors read and approved the final manuscript.

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Table 1 Semiquantitative adipogenic differentiation score

Two criteria scoring system for semiquantitative evaluation of adipogenic differentiation following the oil red O staining procedure. A maximum score of 6 points was possible, with a maximum of 3 points for each criterion.

% of differentiated cells among all MSCs in field of view (10x magnification)		**Size and arrangement of lipid droplets**	
0 points	0 – 5%	0 points	No lipid droplets
1 point	>5 – 50%	1 point	Predominantly few isolated and small-sized (< 1/3 of nucleus diameter) lipid droplets
2 points	>50 – 80%	2 points	Predominantly medium-sized (approximately 1/3 of nucleus diameter) lipid droplets, surrounding the nucleus
3 points	>80 - 100%	3 points	Predominantly large-sized (> 1/3 of nucleus diameter) lipid droplets, merging around the nucleus

Table 2 Gene primer sequences used for qPCR

List of genes analysed by real-time quantitative PCR. GenBank accession numbers of the sequences used for primer design with Primer3 free online software as well as primer sequences and length of the amplicons in base pairs are shown. The sequences of primers of the gene scleraxis were kindly provided by the Institute of Anatomy, University of Veterinary Medicine, Foundation, Hannover, Germany. (F: forward, R: reverse).

Gene	**Primer sequence**	**Amplicon size**
GAPDH	F: CCAGAACATCATCCCTGCTT	158
NM_001163856	R: CGTATTTGGCAGCTTTCTCC	
Collagen 1A2	F: GAAGATGGTCACCCTGGAAA	177
XM_001492939	R: AGGTTCACCCTTCACACCTG	
Scleraxis	F: ACAGAAAGACGGCGATTCGGAGTT	207
NM_001105150	R: AAAGTTCCAGTGGGTCTGGGCAA	

Table 3 MSC yield per gram per days for each tissue type

Data are presented as median (IQR).

(AT-MSC: adipose tissue-derived MSC; di-MSC: MSC isolated by digestion method; ex-MSC: MSC isolated by explant technique; IQR: interquartile range; MSC: multipotent mesenchymal stromal cell; SDFT-MSC: tendon-derived MSC; UCM-MSC: umbilical cord matrix-derived MSC).

* indicates significance with a p-value ≤ 0.05.

	AT-MSC		
	di-MSC	**ex-MSC**	
days in primary culture	6 (2)	11 (5)	*
MSC yield per gram	21.13×10^5 (18.00×10^5)	0.79×10^5 (1.36×10^5)	*
MSC yield per gram per days	352.2×10^3 (180.9×10^3)	13.2×10^3 (12.7×10^3)	*
	SDFT-MSC		
	di-MSC	**ex-MSC**	
days in primary culture	7 (2)	10.5 (1)	*
MSC yield per gram	17.49×10^5 (10.72×10^5)	1.62×10^5 (2.05×10^5)	*
MSC yield per gram per days	291.5×10^3 (176.4×10^3)	17.3×10^3 (19.6×10^3)	*
	UCM-MSC		
	di-MSC	**ex-MSC**	
days in primary culture	10 (4.25)	18 (4)	*
MSC yield per gram	4.16×10^5 (12.66×10^5)	0.70×10^5 (0.66×10^5)	*
MSC yield per gram per days	61.7×10^3 (134.2×10^3)	4.2×10^3 (4.1×10^3)	*

Table 4 Migration and differentiation potential of MSCs

Data are presented as median (IQR).

(AT-MSC: adipose tissue-derived MSC; di-MSC: MSC isolated by digestion method; ex-MSC: MSC isolated by explant technique; IOD: index of osteogenic differentiation; IQR: interquartile range; MSC: multipotent mesenchymal stromal cell; SDFT-MSC: tendon-derived MSC; UCM-MSC: umbilical cord matrix-derived MSC).

	AT-MSC	
	di-MSC	**ex-MSC**
Migration area [mm^2]	0.237 (0.033)	0.207 (0.384)
Adipogenic differentiation score	5.5 (0.0)	5.5 (0.5)
IOD (day 21)	2.2418 (0.6207)	2.0118 (1.5715)
IOD (day 35)	3.7193 (1.5035)	4.8596 (3.3090)
Chondrogenic differentiation score	2.5 (0.75)	3.0 (0.5)
	SDFT-MSC	
	di-MSC	**ex-MSC**
Migration area [mm^2]	0.353 (0.213)	0.238 (0.126)
Adipogenic differentiation score	5.5 (0.0)	5.5 (0.0)
IOD (day 21)	2.8386 (1.2352)	3.0644 (1.7268)
IOD (day 35)	4.2629 (1.4771)	3.7185 (1.8768)
Chondrogenic differentiation score	1.875 (2.75)	3.5 (2.5)
	UCM-MSC	
	di-MSC	**ex-MSC**
Migration area [mm^2]	0.090 (0.124)	0.048 (0.128)
Adipogenic differentiation score	4.5 (1.0)	4.25 (0.75)
IOD (day 21)	1.0211 (0.2223)	1.1055 (0.2843)
IOD (day 35)	1.2135 (0.2767)	0.9519 (0.0800)
Chondrogenic differentiation score	3.5 (1.5)	3.0 (2.75)

Figure 1 Explant technique.

Single cells (white arrows) migrated from the margin of the tissue piece (tendon) and adhered onto the plastic surface to form cell colonies. Following several days in culture, cells developed typical spindle-shaped morphology. Scale bar = 100 μm.

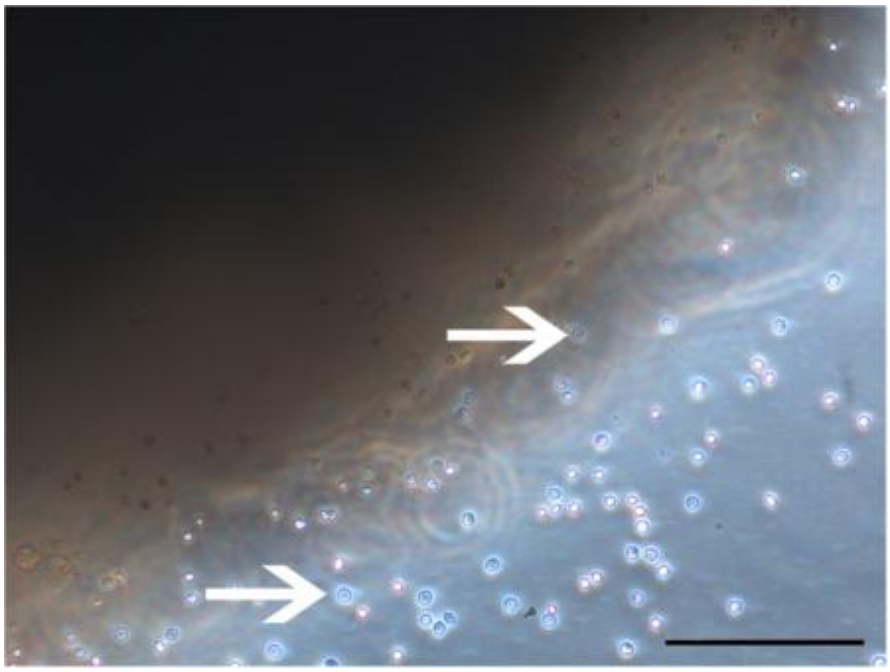


Figure 2 Migration assay.

(a) Culture of MSCs using a spheroid system. **(b)** Attachment of spheroid 4 hours after seeding **(c)** and MSC migration 24 hours after spheroid seeding. **(d)** At the 24 hour time point, the spheroid size was measured (area B) and subtracted from the area of the maximum range of migrated MSCs (area A). Scale bar = 200 μm. (MSCs: multipotent mesenchymal stromal cells).

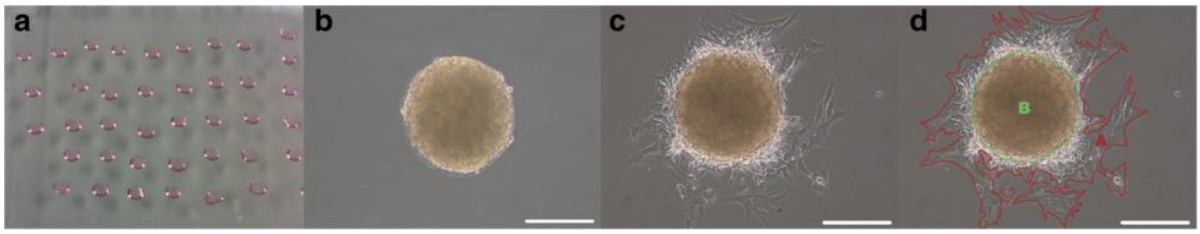


Figure 3 Generation times of MSCs from passages 1 to 7 for each tissue type.

Circle indicates mild outlier; asterisk indicates extreme outlier. No significant differences were observed between di-MSCs and ex-MSCs for each tissue type (p-values > 0.05). Generation time = (cell culture time)/(population doubling). (**a)** AT-MSC: adipose tissue-derived MSC; MSC: multipotent mesenchymal stromal cell; **b)** SDFT-MSC: tendon-derived MSC; **c)** UCM-MSC: umbilical cord matrix-derived MSC).

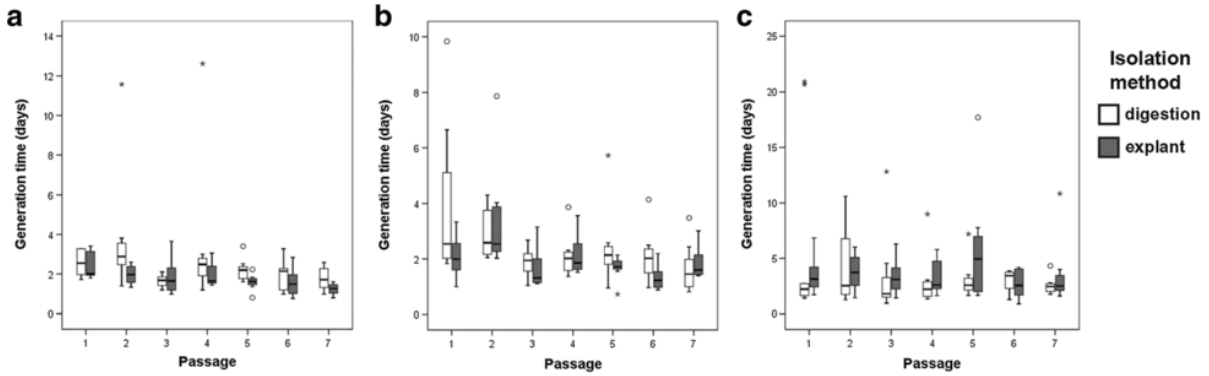


Figure 4 Proliferation rates of MSCs (passages 3 and 8) for each tissue type.

Circle indicates mild outlier. No significant differences were observed between di-MSCs and ex-MSCs for each tissue type (p-values > 0.05). (**a)** AT-MSC: adipose tissue-derived MSC; MSC: multipotent mesenchymal stromal cell; **b)** SDFT-MSC: tendon-derived MSC; **c)** UCM-MSC: umbilical cord matrix-derived MSC).

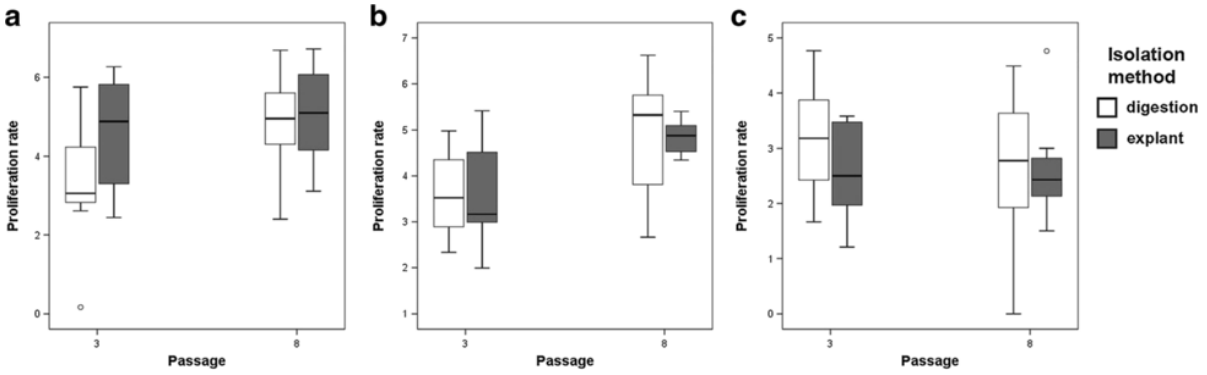


Figure 5 Adipogenic differentiation.

Adipogenic differentiation demonstrated by the accumulation of intracellular lipid droplets stained by oil red O, shown for MSCs derived from tendon tissue (at 3 days of incubation). **(a)** MSCs isolated by digestion. **(b)** MSCs isolated by explant technique. Scale bar = 200 μm. (MSC: multipotent mesenchymal stromal cell).

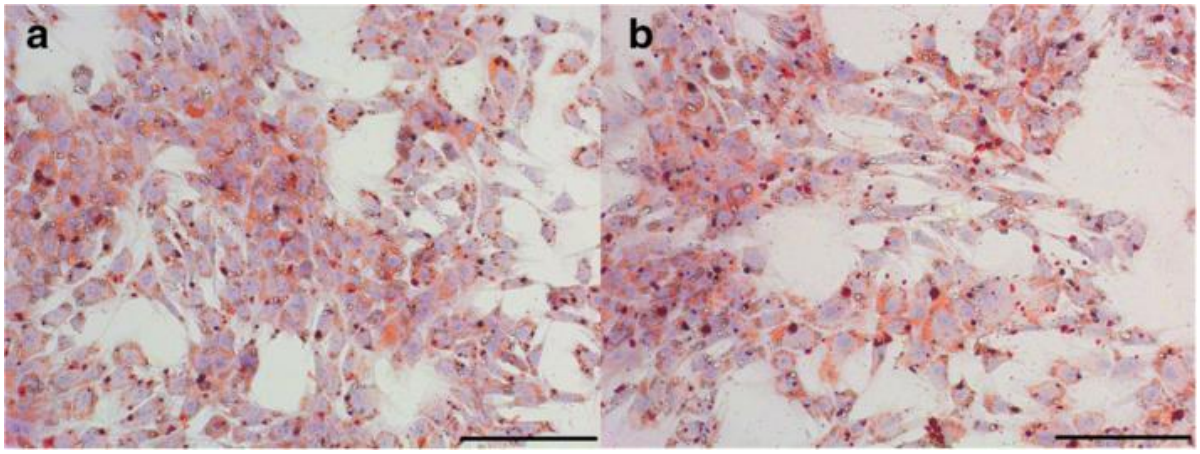


Figure 6 Osteogenic differentiation.

Osteogenic differentiation with deposition of extracellular calcium visualized by von Kossa staining, shown for MSCs derived from adipose tissue (top: 21 days of incubation; bottom: 35 days of incubation). **(a)** MSCs isolated by digestion. **(b)** MSCs isolated by explant technique. Scale bar = 200 µm. (MSC: multipotent mesenchymal stromal cell).

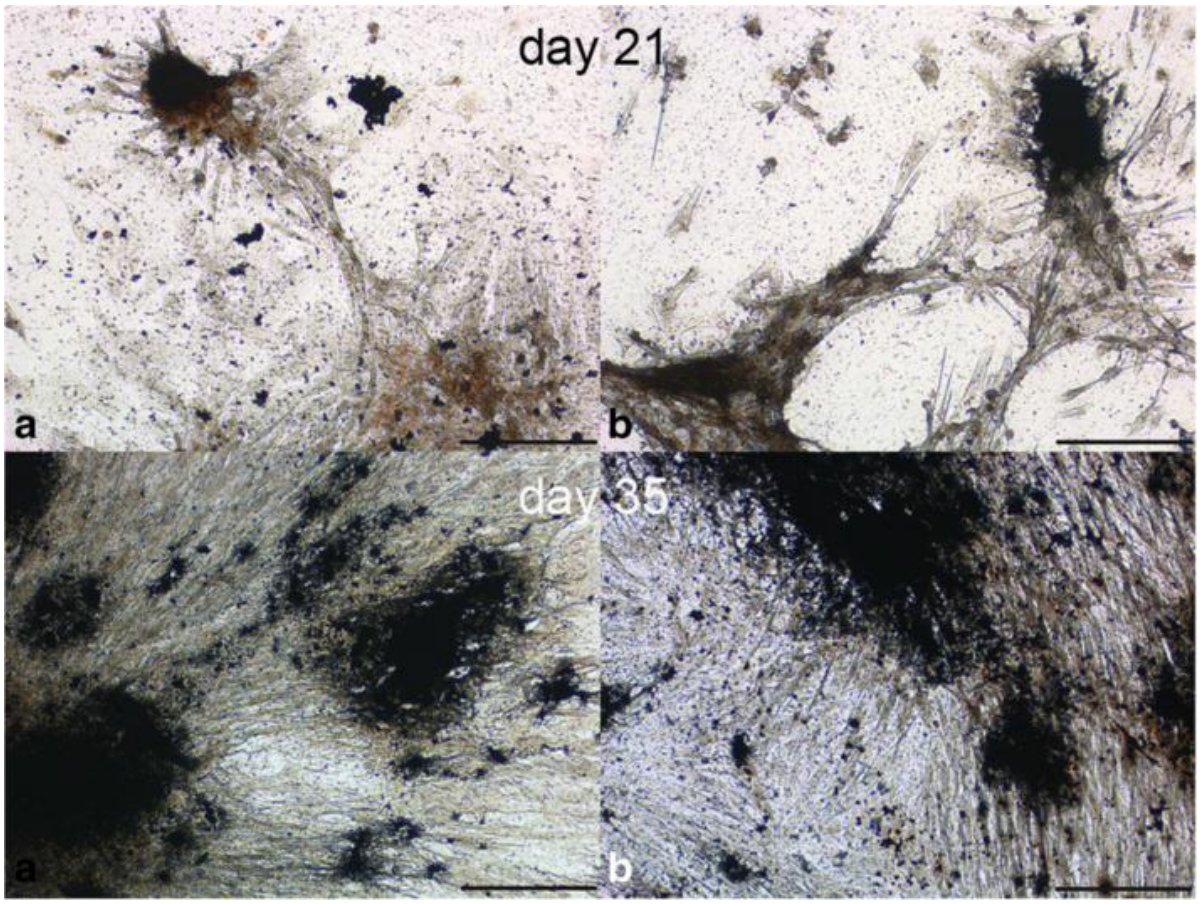


Figure 7 Chondrogenic differentiation.

Chondrogenic differentiation demonstrated by the presence of glycosaminoglycans and collagen. (top) Alcian Blue, (middle) Masson's Trichrome and (bottom) Safranin O staining in MSCs derived from umbilical cord matrix after 21 days of incubation. **(a)** MSCs isolated by digestion. **(b)** MSCs isolated by explant technique. Scale bar = 200 µm. (MSC: multipotent mesenchymal stromal cell).

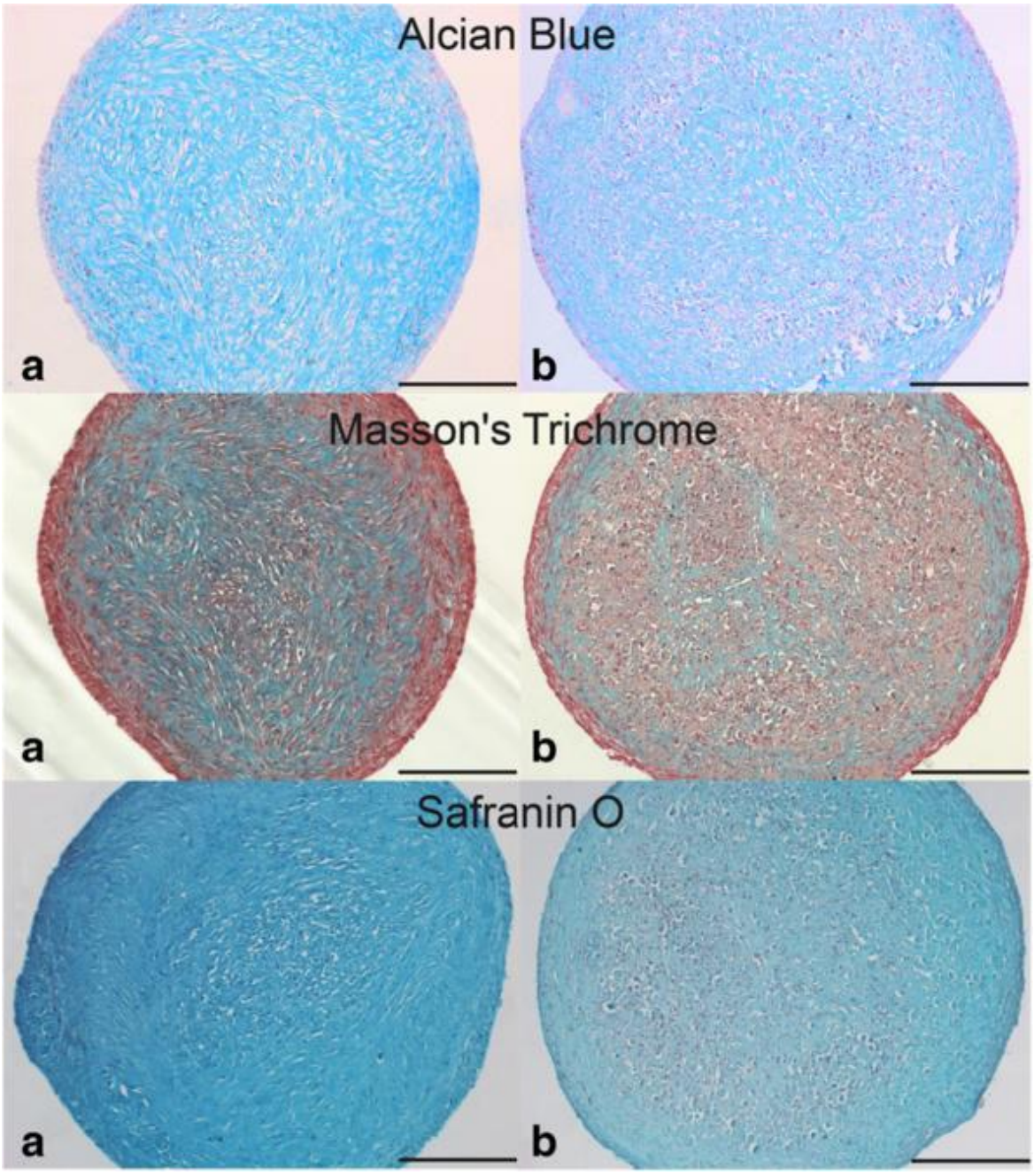
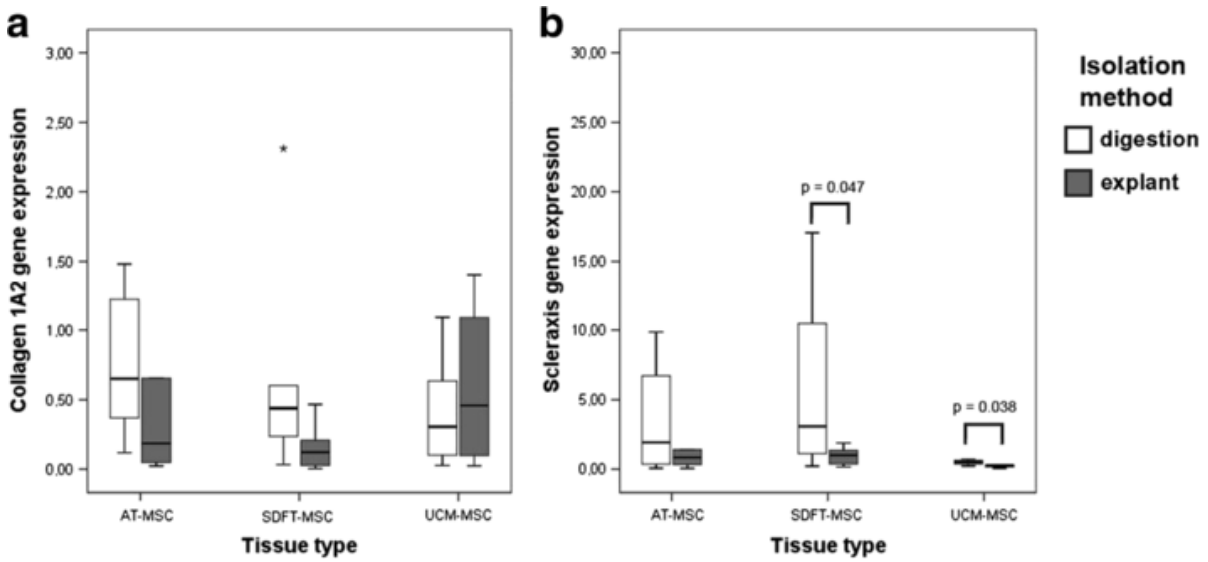


Figure 8 Expression levels of a) collagen 1A2 (Col1A2) and b) scleraxis (Scx).

Star indicates extreme outlier. (AT-MSC: adipose tissue-derived MSC; MSC: multipotent mesenchymal stromal cell; SDFT-MSC: tendon-derived MSC; UCM-MSC: umbilical cord matrix-derived MSC).



4.2 Publikation 2 (Vergleichende Charakterisierung equiner MSCs verschiedener Quellen)

Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources

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Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources ★

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★Preliminary results were presented as a poster at the World Conference on Regenerative Medicine, Leipzig, Germany, december 2011 (Burk et al., 2011).

Abstract

Multipotent mesenchymal stromal cells (MSCs) are a promising therapeutic tool for the treatment of equine tendon and other musculoskeletal injuries. While bone marrow is considered the ‘gold standard’ source of these cells, various other tissues contain MSCs with potentially useful features. The aim of this study was to compare clinically relevant characteristics of MSCs derived from bone marrow, umbilical cord blood and tissue and from adipose tissue and tendon. Cell yield, proliferation, migration, tendon marker expression and differentiation into adipocytes, chondrocytes and osteoblasts was assessed, quantified and compared. Numbers of MSCs obtained from adipose, tendon or umbilical cord tissue were 222-fold higher than those obtained from bone marrow or cord blood. Those cells derived from tendon and adipose tissue exhibited most rapid proliferation. Osteogenic differentiation was most prominent in MSCs derived from bone marrow, and was weak in MSCs derived from umbilical cord blood and tissue. In contrast, the highest levels of chondrogenic differentiation were observed in MSCs derived from these sources. Collagen 1A2 expression was highest in adipose- and tendon-derived MSCs, while scleraxis expression was highest in cord blood- and in tendon-derived MSCs. The findings indicate that MSCs from different sources display significantly diverse properties, which may impact on their therapeutic application.

Keywords: Horse; Mesenchymal stromal cell (MSC); Differentiation; Tendon

Introduction

The treatment of equine orthopaedic conditions with mesenchymal stromal cell (MSC)-based regenerative therapies has gained growing attention in the last decade. Evidence to date suggests these cells improve regeneration in tissues with poor healing properties through their ability to: self-renew; differentiate towards several mesodermal lineages; supply growth factors; and release immunomodulatory cytokines (Richardson et al., 2007; Stewart and Stewart, 2011). Various experimental and clinical studies have demonstrated that the treatment of tendon injuries with MSCs results in significantly improved re-arrangement of the collagen fibres and considerably reduces re-injury rates compared to conventional treatment (Smith et al., 2003; Pacini et al., 2007; Nixon et al., 2008; Smith, 2008; Schnabel et al., 2009; Crovace et al., 2010; Godwin et al., 2011). Furthermore, some studies suggest a beneficial effect of MSCs in the treatment of osteoarthritis (Wilke et al., 2007; McIlwraith et al., 2011).

Currently, bone marrow (BM) and adipose tissue (AdT) are the most commonly used sources of MSCs in equine regenerative medicine. However, MSCs with promising features have also been identified in other tissues (Ribitsch et al., 2010), and taking advantage of these alternative sources might further improve MSC therapy.

Equine MSCs derived from umbilical cord blood (UCB) or tissue (UCT) were first characterised by Koch et al. (2007) and Hoynowski et al. (2007), respectively. In addition to the advantage of non-invasive collection, it is hypothesised that, because these umbilicus-derived cells express markers associated with an embryonic phenotype (Hoynowski et al., 2007; Reed and Johnson, 2008), they may provide a pool of more primitive progenitor cells with broader differentiation capacities (Moretti et al., 2010). Furthermore, human UCB-MSCs have longer telomeres than BM-MSCs and might therefore have a longer lifespan than other adult MSCs (Kogler et al., 2004).

Tendon-derived MSCs (Td-MSCs) may be more similar to the tenocyte phenotype than MSCs derived from other sources, potentially making them suitable for the treatment of tendon injuries. Stewart et al. (2009) demonstrated that when seeded on cell-free tendon scaffolds, the viability and collagen III mRNA expression of equine tendon-derived cells were greater than their BM-derived equivalents, although the trilineage differentiation potential of these cells was not investigated in this study. In mice and rabbits, Td-MSCs can form tendon-like tissue in vivo, in addition to being able to differentiate into adipocytes, osteoblasts and

chondrocytes in vitro (Salingcarnboriboon et al., 2003; Bi et al., 2007; Zhang and Wang, 2010). Although recently, cells isolated from equine tendons have been shown to display the capacity to trilineage differentiation (Lovati et al., 2011a), studies on equine Td-MSCs remain rare, despite the intensive research into the use of MSCs in the treatment of tendon injuries in the horse (Smith et al., 2003; Pacini et al., 2007; Nixon et al., 2008; Smith, 2008; Schnabel et al., 2009; Crovace et al., 2010; Godwin et al., 2011).

While studies have confirmed the in vitro multipotency of equine MSCs derived from different sources, controversy exists concerning the proliferative capacity, life-span, and trilineage differentiation potential of various MSC cell lines (Toupadakis et al., 2010; Lovati et al., 2011b; Vidal et al., 2011), and comprehensive comparative studies are rare. Furthermore, to our knowledge, no studies comparing the tendon regeneration potential of MSCs from different sources have been published. Similarly, although considered to be central to the successful 'homing' and engraftment of MSCs in vivo (Li et al., 2009), the migration potential of equine MSCs derived from different sources has not been assessed. The aim of this study was to comparatively characterise equine MSCs from BM, AdT, UCB, UCT and TdT, with an emphasis on the assessment of properties such as cell proliferation, migration, differentiation and tendon marker expression, which might influence the outcome of MSC therapy.

Materials and methods

Sample collection

Samples of UCB and UCT were collected from 12 foals immediately after birth at the Saxonian Horse Stud (Saechsisches Hauptgestuet), Graditz, Germany, and at The Large Animal Clinic for Theriogenology and Ambulatory Services, University of Leipzig, Germany. The umbilical cord was clamped, the cord vein was punctured and UCB was collected into heparinised syringes (500 IU Heparin-Na/mL, B. Braun Melsungen AG). Subsequently, a 10 cm length of the umbilical cord was obtained, washed in ethanol, iodine and saline solution, and placed in PBS (PAA Laboratories GmbH), supplemented with 500 IU/mL penicillin, 0.5 mg/mL streptomycin (5% penicillin-streptomycin, PAA Laboratories GmbH), 0.05 mg/mL gentamycin (Invitrogen) and 2.5 µg/mL amphotericin (Invitrogen) for transportation.

Bone marrow was collected from 12 adult horses (median age, 4.5 years; interquartile range, 11.0) according to standard surgical procedures. Briefly, the horses were sedated, the sternal region was prepared aseptically and following local anaesthesia, the sternum was punctured with an 11 G bone marrow aspiration needle and a sample was aspirated into a heparinised syringe. Seven of these horses were subsequently euthanised, 6 g of SC AdT was harvested from the supragluteal region, and a 5 cm length of TdT was taken from the mid-metacarpal region of the superficial digital flexor tendon.

Samples were stored at room temperature and processed within 24 h. All procedures were approved by the local ethics committee (Landesdirektion Leipzig, A 13/10).

Isolation and culture of MSCs

Mononuclear cells (MNCs) were separated from UCB and BM by standard density gradient centrifugation (327 *g* at 20 °C for 30 min) using a polysaccharide solution (Ficoll-Paque Premium, GE Healthcare). Prior to processing the solid tissue samples (UCT, AdT, and TdT), UCT was dissected from the cord vessels and TdT was separated from the paratenon. Subsequently, tissues were minced and digested in a collagenase I solution (Invitrogen) (Table 1). Isolated MNCs were seeded into culture flasks containing low concentration glucose (1 g/L) Dulbecco's modified eagle medium (DMEM) (Invitrogen), supplemented with 20% fetal bovine serum (FBS) (Sigma Aldrich), 100 IU/mL penicillin, 0.1 mg/mL streptomycin (1% penicillin-streptomycin) and 0.05 mg/mL gentamycin. The seeding density of MNCs was approximately 500,000 MNCs/cm^2 for UCB- and BM-MNCs and approximately 50,000 MNCs/cm^2 for MNCs from the solid tissues, respectively. For the UCB and UCT cell cultures, 0.5 µg amphotericin /mL of medium was added until first passaging.

Cells were allowed to attach for 2 days under standard culture conditions (37 °C and 5% CO_2), and were then washed with PBS to remove non-adherent MNCs. Medium was changed twice weekly until the colonies were confluent, at which time MSCs were trypsinised. After first passaging, MSCs from eight UCB, eight UCT, 10 BM and seven AdT and TdT samples were used for all further experiments. The remaining samples were used in experiments unrelated to this study.

Expansion and proliferation assays

To assess MSC expansion and generation times (GTs), passage (P) 1 to P7 MSCs from all sources were seeded at a density of 3,000 MSCs/cm^2. Cell cultures were checked daily and

the medium changed twice weekly, until cells were 80% confluent and MSCs were passaged by trypsinisation. Population doubling rates (PDs) and GTs were calculated as follows:

PD = (cell count harvest/cell count seeding)/ln 2
GT = 1/(PD/days in culture)

As some samples underwent senescence (defined as having a negative PD before P8), a Kaplan-Meier analysis of sample survival was performed, and only non-senescent samples were included in the PD and GT calculations. At P3 and P8, a tetrazolium (MTS) assay (Cell Titer 96 Aqueous One Solution Proliferation Assay, Promega) was performed to evaluate cell proliferation rates (PRs). The MSCs were seeded in 96 well plates (1000 cells/well). At days 1 and 7, MTS reagent was added and formazan production was assessed photometrically, according to the manufacturer's instructions. PRs were calculated using the following formula:

PR = optical density day 7/optical density day 1

Spheroid culture and migration assay

Passage 3 MSCs were cultured in 'hanging drops' (5000 cells/drop) for 24 h until spheroid formation was observed. Spheroids were then harvested, seeded and cultured under standard conditions, which allowed MSC migration from the spheroids onto the culture plates. After another 24 h, the migration area was determined (Fig. 1).

Trilineage differentiation assays

For adipogenic differentiation, P3 MSCs were incubated in adipogenic differentiation medium (DMEM-F12 [PAA Laboratories GmbH], supplemented with 15% rabbit serum, 1 µM dexamethasone, 100 µM indomethacin, 500 µM 3-isobutyl-1-methylxanthine, 700 nM bovine insulin [all Sigma Aldrich], and antibiotics) for 3 days. The intensity of adipogenic differentiation was assessed by two 'blinded' observers using a scoring system based on Oil Red O staining (Gittel et al., 2011) (Table 2). For osteogenic differentiation, P3 MSCs were cultured in osteogenic differentiation medium (DMEM-F12 supplemented with 10% FBS, 100 µM L-ascorbate-2-phosphate, 10 mM β-glycerophosphate, 100 nM dexamethasone [all Sigma Aldrich] and antibiotics) for 21 and 35 days, followed by von Kossa staining. For positively-stained samples, the index of osteogenic differentiation (IOD) was determined as described by Ostanin et al. (2008). Briefly, optical densities of stained differentiated and

control samples were measured photometrically (λ=492 nm) and the IOD calculated as follows:

IOD = optical density (differentiated)/optical density (control).

Chondrogenic differentiation of P3 MSCs was performed in pellet culture as described by Giovannini et al. (2008). Chondrogenic differentiation medium consisted of high concentration glucose (4.5 g/L) DMEM (PAA Laboratories GmbH), 10 ng/mL human TGF-β1 (Acris Antibodies), 1% ITS+ premix (BD Biosciences), 100 nM dexamethasone, 100 µM L-ascorbate-2-phosphate, 400 µM proline (Sigma Aldrich) and antibiotics. After incubation for 21 days, paraffin sections were prepared and stained with Alcian blue and Masson's Trichrome stains. Qualitatively positive samples were further stained with Safranin O and evaluated by two 'blinded' observers using the 'Bern score' based on three criteria: uniformity and darkness of the Safranin O stain; distance between cells and amount of matrix accumulated; and cell morphology (Grogan et al., 2006).

Non-induced MSCs from different sources were used as negative controls for each of the stains (data not shown).

Real Time RT-PCR

mRNA from the P3 MSCs was isolated and transcribed into cDNA using commercially available kits (RNeasy Mini Kit and Omniscript RT Kit, Qiagen), according to the manufacturer's instructions. Primers for GAPDH (forward, 5'-CCAGAACATCATCCCTGCTT-3'; reverse, 5'- CGTATTTGGCAGCTTTCTCC-3') and collagen 1A2 (forward, 5'-GAAGATGGTCACCCTGGAAA-3'; reverse, 5'-AGGTTCACCCTTCACACCTG-3') were designed using Primer3 online software. The scleraxis primers (forward, 5'-ACAGAAAGACGGCGATTCGGAGTT-3'; reverse, 5'-AAAGTTCCAGTGGGTCTGGGCAA-3') had previously been evaluated at the Institute of Anatomy, University of Veterinary Medicine, Foundation, Hannover. Quantitative RT-PCR was performed using a SYBR green master mix (iQ SYBR Green Supermix, Bio-Rad Laboratories). Genes were amplified in 40 cycles of 90 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s (7500 Real-Time PCR System, Applied Biosystems). Copy numbers were obtained from standard curves and normalised to GAPDH RNA expression.

Statistical analysis

Kruskal-Wallis one way analyses of variance and subsequent Mann-Whitney *U* tests were performed to analyse differences among the sample groups (IBM PASW Statistics 18 software). The level of significance was set at $\alpha = 0.05$.

Results

Sample collection and cell yield

Samples of UCB, UCT and BM were collected without adverse effects to the donors. Although a lower volume of BM was collected and processed compared to UCB, significantly higher total MNC yields were obtained from BM ($P<0.01$). Furthermore, the numbers of MNCs obtained/g solid tissue (UCT, AdT, and TdT) varied significantly, with AdT providing the highest and UCT the lowest numbers (UCT vs. AdT, $P<0.001$; UCT vs. TdT, $P<0.01$) (Table 3). The isolation of MSCs with a fibroblast-like phenotype was achieved in all samples, with significant differences between the P0 culture times ($P<0.01$). The MSC yield/seeded MNCs/days of culture (P0) was similar between UCB and BM samples and also within the solid tissue samples. However, the MSC yield from solid tissues was 222-fold higher than the yield from UCB and BM ($P<0.001$) (Table 3). Two UCT-MSC samples had to be discarded due to bacterial or fungal contamination which became evident after MNC seeding.

Proliferation and migration of MSCs

The MSCs from all sources exhibited short GTs at P1, indicating rapid proliferation. With increasing passages, UCB- and BM-MSCs displayed lower replication rates while Ad-MSCs and Td-MSCs continued to proliferate rapidly, reaching P8 in a shorter period of time ($P<0.01$) (Table 3). Furthermore, 6/8 UCB-MSC samples and 7/10 BM-MSC samples underwent senescence before P8 (Fig.2). Significant differences between the GTs of the different sample types were evident at P2, P3, P4 and P6, respectively ($P<0.05$) (Fig.3). Proliferation rates, determined by MTS assays, supported the results obtained by calculation of PDs and GTs. However, differences in PRs were only significant at P8 ($P<0.05$) (Fig.3).

MSCs from all sources formed spheroids when cultured in hanging drops and migrated from the spheroids after seeding. Ad- and Td-MSCs migrated faster than UCT- and BM-MSCs, covering a significantly larger area on the culture dish within 24 h ($P<0.01$) (Table 4).

Trilineage differentiation

Cells from all sources contained intracellular lipid droplets, as visualised by Oil Red O staining, indicating early-stage adipogenic differentiation (Fig.4). There were no significant differences between the different sample types, except between Td- and UCT-MSCs ($P<0.05$) (Table 4). Osteogenic differentiation, marked by extracellular calcium deposition (Fig.4), was pronounced in BM-, Ad- and Td-MSCs at day 21 and increased further until day 35. In contrast, osteogenic differentiation was weak in UCB- and UCT-MSCs, and only UCT-MSCs showed a slight increase in this form of differentiation between day 21 and 35. In one UCT-MSC sample calcium deposition was not detectable at any timepoint. Median IOD values supported this observation and were significantly higher in BM-, Ad- and Td-MSCs at day 21 ($P\leq0.01$) and 35 ($P<0.05$) (Table 4).

Chondrogenic differentiation (Fig.5), as determined by Alcian blue and Masson's trichrome staining, was evident in all UCB- and UCT-MSCs, but only in 6/10 BM-MSC, 5/7 Ad-MSC, and 6/7 Td-MSC samples, respectively. The evaluation of Safranin O-stained sections of these qualitatively positive samples using the Bern score revealed that UCB-MSCs displayed the highest chondrogenic differentiation capacity, followed by UCT-MSCs, whereas BM-MSCs scored lowest. However, differences in this score were only significant between UCB- and BM-MSCs or Td-MSCs ($P<0.05$ in each case) (Table 4).

Tendon marker expression

All MSC samples exhibited basal gene expression of collagen 1A2 and scleraxis (Fig.6). The highest levels of collagen 1A2 expression were found in Ad-MSCs, followed by Td-MSCs, whereas BM-MSCs had the lowest expression of collagen 1A2. Differences were significant between: Ad-MSCs and BM-MSCs ($P<0.01$); Ad-MSCs and UCB-MSCs ($P<0.05$); and between Td-MSCs and BM-MSCs ($P<0.05$). Scleraxis was expressed at the highest levels in UCB-MSCs, followed by Td-MSCs. Differences in scleraxis expression were significant between UCB-MSCs and UCT-MSCs ($P<0.001$).

Discussion

In this study, we compared the proliferation, migration, trilineage differentiation capacity and expression of tendon markers in equine MSCs derived from BM, AdT, UCB, UCT and TdT. Our results reveal significant differences between MSCs derived from these sources, indicating potential advantages and disadvantages for the use of each type of MSCs in particular clinical applications.

The number of readily available, viable MSCs plays a crucial role with regard to treatment success when autologous MSCs are used, as successful treatment requires the application of a sufficient quantity of MSCs shortly after the injury has occurred (Godwin et al., 2011). In addition to their importance in direct therapy, high cell yields and viability also facilitate the cryopreservation of these cells.

The present study was successful in isolating fibroblast-like MSCs using standard protocols in all samples, irrespective of their source. However, MSC yields and proliferation were significantly higher in all solid tissues (UCT, AdT, and TdT) compared to BM and UCB. As not all samples used in this study were donor-matched, it should be considered that there might have been donor age-related effects on cell yield and proliferation. However, donor age did not correlate with cell yields or GTs (data not shown). Furthermore, our findings are consistent with a recently published study by Vidal et al. (2011) which specifically investigated population doubling and senescence of BM-, Ad- and UCT-MSCs, including the assessment of senescence markers and telomere lengths. Kern et al. (2006) reported that senescence ratios of human MSCs in early passages were highest in UCB-MSCs and lowest in Ad-MSCs. Interestingly, in the same study, UCB-MSCs that had not undergone early senescence replicated most rapidly and had the longest lifespan.

The ability of MSCs to migrate is fundamental to their systemic application (Li et al., 2009), and in supporting graft integration in local therapies. Guest et al. (2010) showed that BM-MSCs remained close to their site of injection in artificially-induced tendon lesions, unlike embryonic stem cells, which became widely distributed in the surrounding tissue. In the current study, we demonstrated that Td-, Ad- and UCB-MSCs migrate faster than BM-MSCs, suggesting their graft integration in vivo may be enhanced. We chose to combine migration assays with the spheroid culture of MSCs, as this technique has been shown to enhance differentiation potential (Wang et al., 2009). In clinical cases that do not require a scaffold,

the injection of MSC spheroids might be a feasible approach in introducing viable MSCs with enhanced differentiation potential.

Multilineage potential was evident in all MSC samples, demonstrating the multipotential character of these cells, although not all samples followed all differentiation pathways. Significant differences were found in the osteogenic and chondrogenic differentiation potential of the cells, indicating that the source of the cells used may be important in the treatment of bone or cartilage defects.

As evaluated in previous experiments, UCB-MSCs demonstrated poor adipogenic differentiation when using standard protocols (Gittel et al., 2011), which is in accordance with Koch et al. (2007). Rabbit serum has been reported to enhance adipogenic differentiation (Janderova et al., 2003; Koch et al., 2007; Giovannini et al., 2008). We therefore used a differentiation medium supplemented with rabbit serum, which led to distinct adipogenic differentiation of all MSC samples within 3 days. Incubation with rabbit serum for a longer period, however, can lead to cell detachment (Gittel et al., 2011).

BM-MSCs exhibited particularly intense osteogenic differentiation, while UCB- and UCT-MSCs displayed comparatively little calcium deposition. Our findings support those of Toupadakis et al. (2011), who intensively characterised and compared the osteogenic differentiation potential of equine MSCs from different sources. However, albeit less than that of MSCs derived from other sources, the osteogenic potential of equine UCB- and UCT-MSCs has been shown (Hoynowski et al., 2007; Koch et al., 2007; Reed and Johnson, 2008; Passeri et al., 2009), and UCB-MSCs have formed bone-like matrices within hydroxyappatite scaffolds (Figueroa et al., 2011). Photometric measurement, according to Ostanin et al. (2008), could not detect any differences between differentiated and control samples of UCB- and UCT-MSCs, although only samples showing qualitative evidence of calcium deposition were included. However, as the optical density was significantly increased in differentiated BM-, Ad- and Td-MSC samples compared to their respective controls, the IOD, although not a very sensitive parameter, was considered suitable to roughly quantify differences in osteogenic differentiation capacity.

Chondrogenic differentiation was most prominent in UCB-MSCs, as suggested by Berg et al. (2009). Bone marrow-MSCs displayed the weakest chondrogenic potential in the present study, although in other studies, intense chondrogenic differentiation was observed with equine BM-MSCs (Giovannini et al., 2008), which was even superior to that of Ad-MSCs

(Vidal et al., 2008). Lovati et al. (2011) found evidence of weak chondrogenic differentiation in BM-MSCs but, contrary to our findings, could not induce chondrogenesis in UCT-MSCs. These conflicting results emphasise that the adjustment of culture conditions and differentiation media with respect to the origin of MSCs is essential in achieving optimal chondrogenesis.

One of the reasons this is the first study to compare the tendon regeneration potential of MSCs derived from different sources is the difficulty associated with achieving tenogenic differentiation (Hoffmann and Gross, 2007; Aslan et al., 2008; Butler et al., 2008). To date, there has only been one study that has attempted the tenogenic differentiation of equine MSCs, through exposure to BMP-12 (Violini et al., 2009). Not only is the induction of tenogenic differentiation challenging, but verification of this process is also complex, as there seems to be no clear demarcation between mature tenocytes and fibroblasts. Currently, gene expression analysis of tendon markers is most commonly used to identify tenogenic properties (Chen et al., 2008; Omae et al., 2009; Taylor et al., 2009; Park et al., 2010; Sharma and Snedeker, 2010). However, it must be considered that results obtained at an mRNA level do not always reflect protein expression by the cells in question.

In analysing gene expression markers in different musculoskeletal tissues, Taylor et al. (2009) found high expression of collagen 1A2 and scleraxis in equine tendon, suggesting these markers are suitable in the evaluation of tenogenesis. While the use of collagen 1A2, the most abundant protein in tendon extracellular matrix, as a tendon marker is self-evident, scleraxis plays a crucial role in tendon development (Schweitzer et al., 2001; Shukunami et al., 2006). In the present study, investigating basal tendon marker expression in undifferentiated, monolayer-cultured MSCs, Td-MSCs expressed both important markers, collagen 1A2 and scleraxis, at high levels contemporaneously. This supports the hypothesis that Td-MSCs, while also being capable of adipogenic, osteogenic and chondrogenic differentiation, may be the optimal cell type for MSC-based tendon therapy.

Concerning autologous Td-MSC therapies, donor site morbidity is a major problem, especially when using the superficial digital flexor tendon as a cell source. Nevertheless, for autologous Td-MSC therapy, it is possible to harvest the tissue from less functionally essential tendons, such as the digital extensor tendons. Moreover, in veterinary medicine, allogeneic MSC application as an ‘off-the-shelf’ product has repeatedly been proposed. In the horse, the application of allogeneic BM- and placentally-derived MSCs did not lead to

increased immune responses, compared to the application of autologous MSCs (Guest et al., 2008; Carrade et al., 2011). Furthermore, the clinical outcome of tendon therapy using either allogeneic or autologous Ad-MSCs, was equally favourable (Del Bue et al., 2008). However, the immunological properties of Td-MSCs remain to be investigated.

Analysis of surface marker expression patterns will have to provide further insights into the characteristics of equine MSCs from different sources. So far, the establishment of a uniform set of markers for equine MSCs, which corresponds to the criteria set for human MSCs (Dominici et al., 2006), remains elusive, as most commercially available antibodies do not recognise their corresponding equine epitopes (De Schauwer et al., 2011). However, initial studies in this area have proved promising (Braun et al., 2010, Radcliffe et al., 2010, Ranera et al., 2011; De Schauwer et al., 2012), and provide a platform from which we can further extend our understanding of equine stem cell biology.

Conclusions

Equine AdT contains high numbers of highly viable MSCs with reliable migration and differentiation capacities, and is therefore a convenient cell source for autologous or allogeneic regenerative therapies. However, in specific clinical settings, it may be beneficial to take advantage of MSC source-specific differentiation capacities. Although Td-MSCs may be especially suitable for tendon therapy, further in vivo studies will be required to substantiate our in vitro findings.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1

Conditions for tissue digestion for mononuclear cell isolation from solid tissues. Collagenase I concentrations and incubation times were evaluated in previous experiments (data not shown). Tissue digestion was performed at 37 °C with continuous shaking. UCT, umbilical cord tissue; AdT, adipose tissue; TdT, tendon tissue.

	Collagenase I /mL	**Incubation time**
UCT	2.4 mg	6 h
AdT	0.8 mg	4 h
TdT	5.6 mg	6 h

Table 2

Semi-quantitative scoring system used in the evaluation of adipogenic differentiation of mesenchymal stromal cells (MSCs).

% of differentiated cells of all MSCs in 10x magnification field		**Size and arrangement of lipid droplets**	
0	0 – 5%	0	No droplets
1	>5 – 50%	1	Predominantly isolated and small (<1/3 of nuclear diameter)
2	>50 – 80%	2	Predominantly medium-sized (approximately 1/3 of nuclear diameter) surrounding nucleus
3	>80 - 100%	3	Predominantly large (>1/3 of nuclear diameter) merging around nucleus

Table 3

Cell yields and culture times. Data presented as medians (interquartile range). MSC, mesenchymal stromal cell; MNC, mononuclear cell; UCB, umbilical cord blood; BM, bone marrow; UCT, umbilical cord tissue; AdT, adipose tissue; TdT, tendon tissue.

	Processed sample volume /wet weight	**Mononuclear cell yield total**	**Mononuclear cell yield /tissue unit**	**Days P0**	**MSC yield/ MNCs/days**	**Days until P8 ***
UCB	146 mL (30 mL)	18.7×10^6 (13.0×10^6)	1.1×10^5/mL (0.9×10^5/mL)	11.5 (5.25) [d, e]	1.1×10^{-3} (17.9×10^{-3})	82.5 (16.5) [d, e]
BM	32.5 mL (20 mL)	46.0×10^6 (33.1×10^6) [a]	11.3×10^5/mL (8.6×10^5/mL) [a]	14.0 (3.0) [c, d, e]	1.5×10^{-3} (1.5×10^{-3})	72.0 (32.0) [d, e]
UCT	5.2 g (0.5 g)	0.8×10^6 (1.8×10^6)	1.6×10^5/g (3.4×10^5/g)	10.0 (3.75) [d, e]	275.0×10^{-3} (362.3×10^{-3}) [a, b]	60.0 (26.5)
AdT	5.6 g (0.9 g)	9.7×10^6 (10.3×10^6)	17.8×10^5/g (15.5×10^5/g) [c]	6.0 (1.5)	251.4×10^{-3} (265.3×10^{-3}) [a, b]	59.0 (17.0)
TdT	5.2 g (0.4 g)	3.6×10^6 (2.3×10^6)	6.8×10^5/g (5.7×10^5/g) [c]	7.0 (1.5)	280.6×10^{-3} (216.7×10^{-3}) [a, b]	53.0 (8.5)

[a] significantly higher values compared to UCB.
[b] significantly higher values compared to BM.
[c] significantly higher values compared to UCT.
[d] significantly higher values compared to AdT.
[e] significantly higher values compared to TdT.
* Samples that underwent senescence before P8 not included.

Table 4

Results of migration and trilineage differentiation assays. Data presented as medians (interquartile range). For quantification of osteogenic and chondrogenic differentiation intensity, only samples with qualitative evidence of differentiation were included. MSC, mesenchymal stromal cell; UCB-MSC, umbilical cord blood-derived MSC; BM-MSC, bone marrow-derived MSC; UCT-MSC, umbilical cord tissue-derived MSC; AdT-MSC, adipose tissue-derived MSC; TdT-MSC, tendon-derived MSC.

	Migration area (mm^2)	**Adipogenic differentiation score**	**Osteogenic differentiation index (day 21)**	**Osteogenic differentiation index (day 35)**	**Chondrogenic differentiation score**
UCB-MSC	0.161 (0.267)	5.0 (1.5)	1.10 (0.48)	0.97 (0.71)	6.000 (4.500) [c, d]
UCT-MSC	0.090 (0.124)	4.5 (1)	1.02 (0.22)	1.21 (0.28)	3.500 (1.500)
BM-MSC	0.097 (0.186)	5.0 (2.25)	3.65 (3.04) [a, b]	6.78 (4.18) [a, b]	1.000 (2.625)
AdT-MSC	0.237 (0.033) [b]	5.5 (0)	2.24 (0.62) [a, b]	3.72 (1.50) [a, b]	2.500 (0.750)
TdT-MSC	0.352 (0.213) [b, c]	5.5 (0) [b]	2.83 (1.24) [a, b]	4.26 (1.47) [a, b]	1.875 (2.750)

[a] significantly higher values compared to UCB-MSCs.
[b] significantly higher values compared to UCT-MSCs.
[c] significantly higher values compared to BM-MSCs.
[d] significantly higher values compared to Td-MSCs.

Fig.1.

Migration assay by spheroid culture of tendon derived mesenchymal stromal cells (MSCs). Attachment of the spheroid 4 h after seeding (a), and MSC migration 12 h (b), and 24 h (c), after seeding, respectively. At 24 h, the area covered by the remaining spheroid (area B) was measured and subtracted from the area covered by migrating MSCs including the spheroid (A), to determine the migration area (d). Scale bars as indicated.

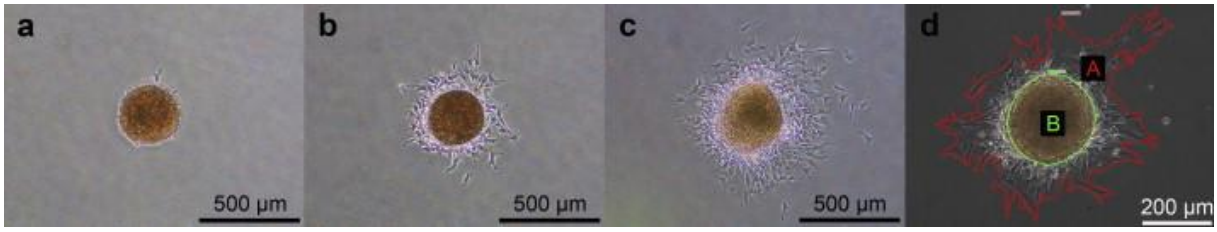


Fig.2.

Kaplan-Meier plot of cell survival (cumulative sample survival vs. time of senescence). MSC, mesenchymal stromal cell; UCB-MSC, umbilical cord blood-derived MSC; BM-MSC, bone marrow-derived MSC; UCT-MSC, umbilical cord tissue-derived MSC; Ad-MSC, adipose tissue-derived MSC; Td-MSC, tendon-derived MSC.

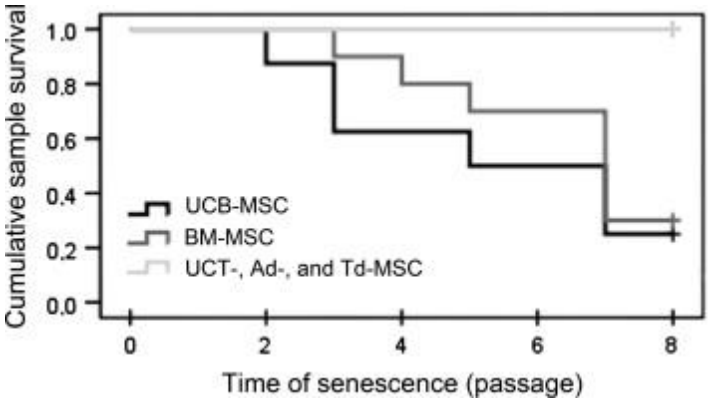


Fig.3.

Left boxplot: generation times (GTs) of mesenchymal stromal cells (MSCs) (passage [P] 1 - 7). Black arrows indicate significantly shorter GTs compared to umbilical cord blood (UCB)-MSCs, light-coloured arrows indicate significantly shorter GTs compared to UCB-MSCs and bone marrow (BM)-MSCs. Right boxplot: proliferation rates of MSCs (P3 and P8). Arrows indicate significantly higher values compared to UCB-MSCs, UCT-MSCs and BM-MSCs. Circle indicates mild outlier; star indicates extreme outier. MSC, mesenchymal stromal cell; UCB-MSC, umbilical cord blood-derived MSC; BM-MSC, bone marrow-derived MSC; UCT-MSC, umbilical cord tissue-derived MSC; Ad-MSC, adipose tissue-derived MSC; Td-MSC, tendon-derived MSC.

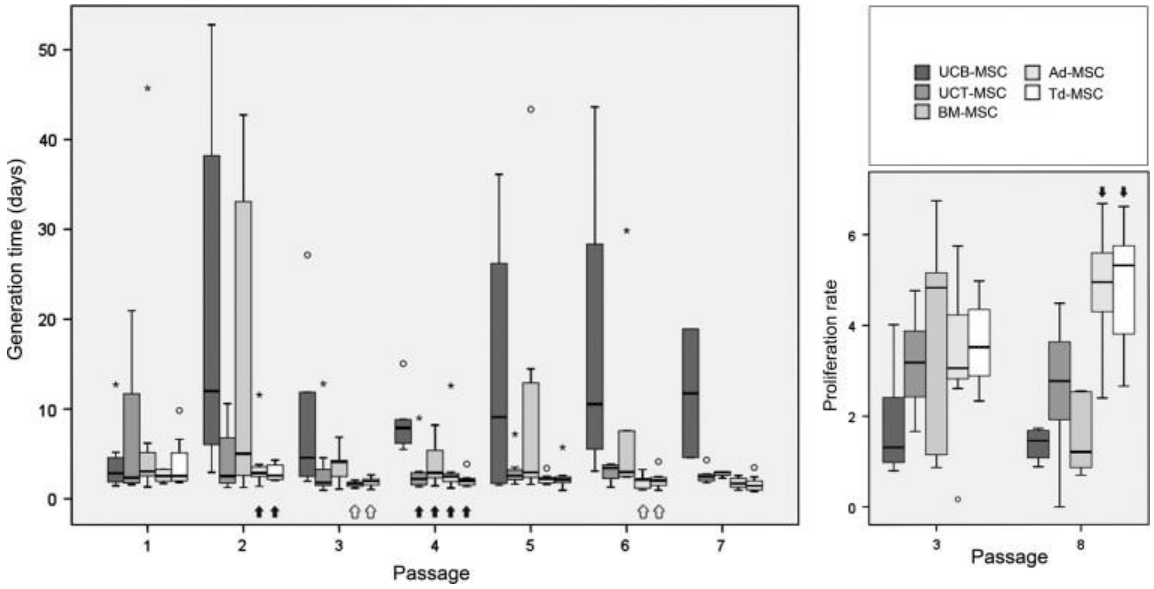


Fig.4.

Representative photographs of adipogenic and osteogenic differentiation. Intracellular lipid droplets following induction of adipogenic differentiation, visualised by Oil Red O staining, and extracellular calcium deposition following induction of osteogenic differentiation, visualised by von Kossa (v. Kossa) staining, respectively. MSC, mesenchymal stromal cell; UCB-MSC, umbilical cord blood-derived MSC; BM-MSC, bone marrow-derived MSC; UCT-MSC, umbilical cord tissue-derived MSC; Ad-MSC, adipose tissue-derived MSC; Td-MSC, tendon-derived MSC. Scale bars as indicated.

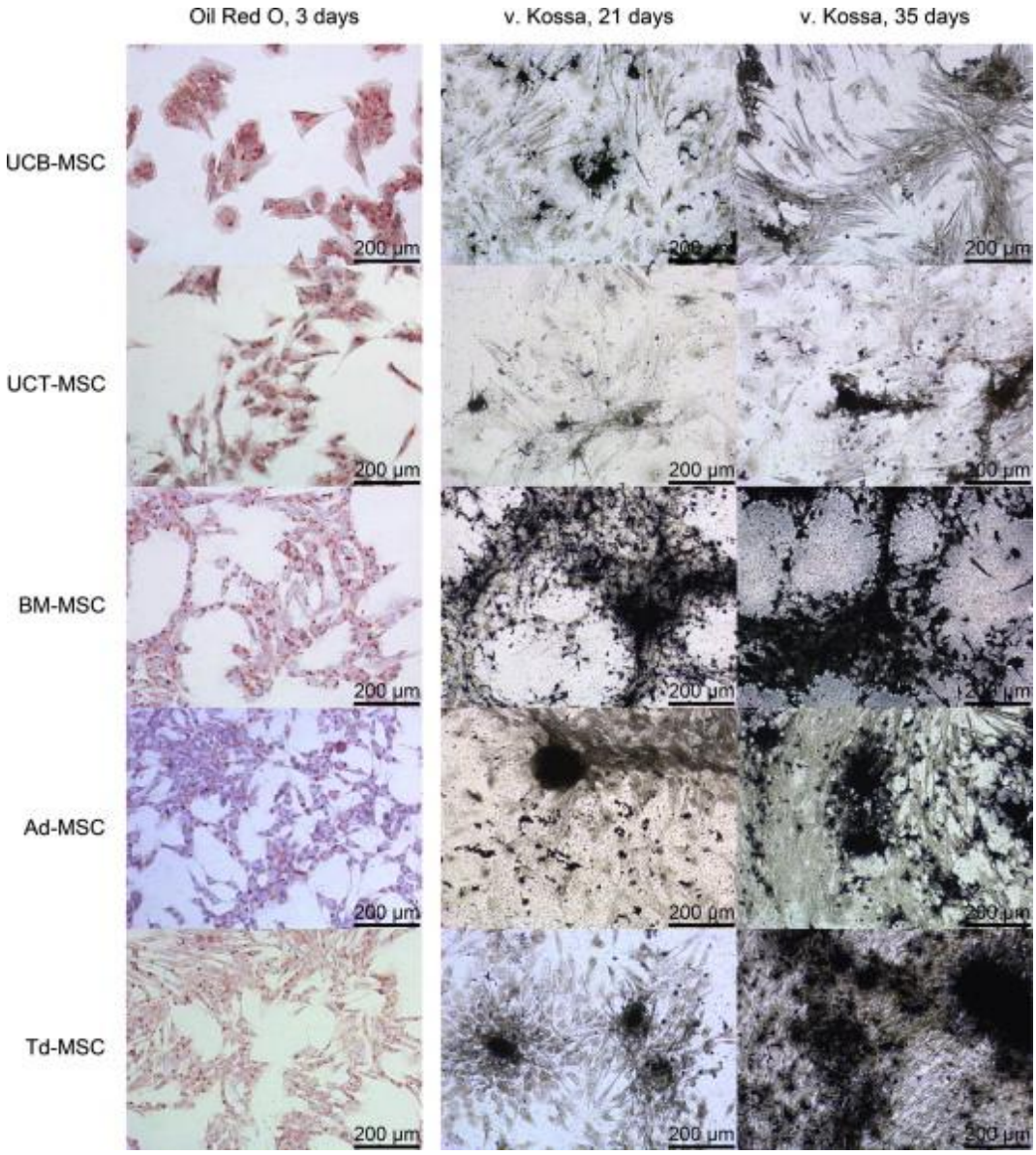


Fig.5.

Representative photographs of chondrogenic differentiation in pellet culture 21 days after induction. Glycosaminoglycans or collagen visualised by Alcian Blue and Safranin O or Masson's trichrome staining, respectively. MSC, mesenchymal stromal cell; UCB-MSC, umbilical cord blood-derived MSC; BM-MSC, bone marrow-derived MSC; UCT-MSC, umbilical cord tissue-derived MSC; Ad-MSC, adipose tissue-derived MSC; Td-MSC, tendon-derived MSC. Scale bars as indicated.

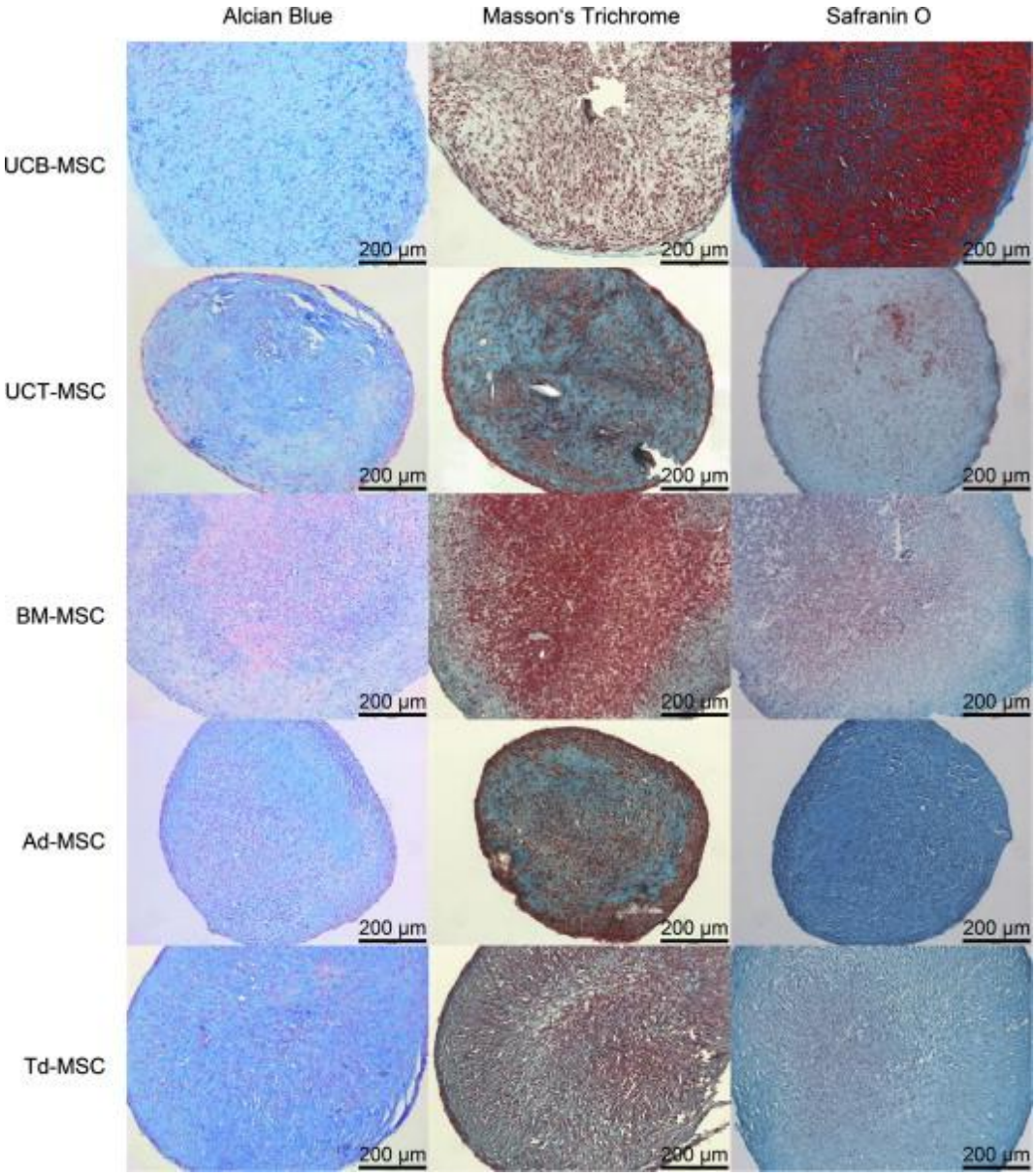
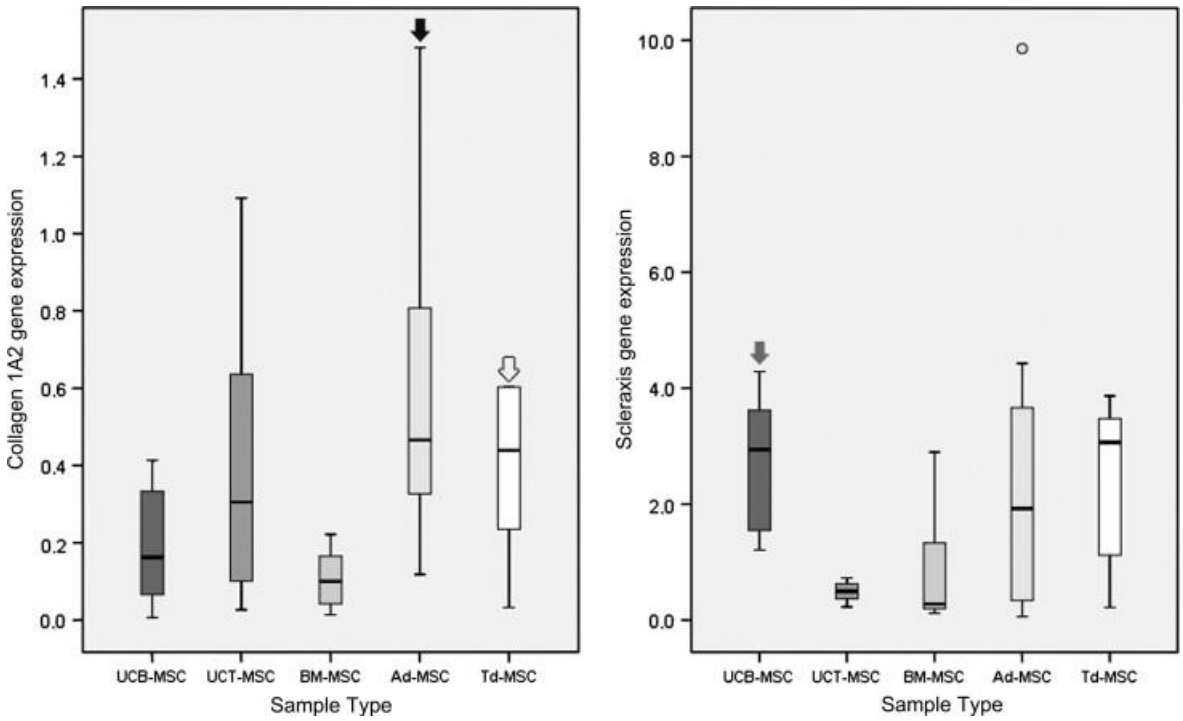


Fig.6.

Gene expression of collagen 1A2 and scleraxis, normalised to GAPDH. Black arrow indicates significantly higher expression compared to umbilical cord blood (UCB)-mesenchymal stromal cell (MSCs) and bone marrow (BM)-MSCs. Light-coloured arrow indicates significantly higher expression compared to BM-MSCs, and grey arrow indicates significantly higher expression compared to umbilical cord tissue (UCT)-MSCs. Circle indicates mild outlier. UCT-MSC, umbilical cord tissue-derived MSC; Ad-MSC, adipose tissue-derived MSC; Td-MSC, tendon-derived MSC.



5 Diskussion

5.1 Material und Methoden

Untersuchungsgegenstand der hier vorliegenden in-vitro-Studie waren equine MSCs. Der erste Teil der Studie beschäftigte sich dabei mit den Einflüssen der Isolationsmethode auf MSCs aus soliden Geweben. Der darauf folgende Teil der Studie war der Vergleich von MSCs aus verschiedenen Geweben. Bisherige umfassende Studien zur Bedeutung der Zellquelle und der Isolationsmethode existierten für MSCs vom Pferd bislang noch nicht. Dennoch ist eine solche Grundlagenforschung für equine MSCs von Bedeutung, da in der Pferdemedizin die Applikation von MSCs unter anderem bei muskuloskelettalen Erkrankungen bereits klinisch angewendet wird (CAPLAN 2009; SMITH 2010). Dies erfolgt auch weitverbreitet bei den natürlich auftretenden Läsionen der oberflächlichen Beugesehne bei der Spezies Pferd (BURK und BREHM 2011; GODWIN et al. 2012; RICHARDSON et al. 2007; SMITH 2008) oder bei Gelenkerkrankungen (MCILWRAITH et al. 2011). Vorteile aus in-vitro- und in-vivo-Studien zum Einsatz von MSCs beim Pferd ergeben sich auch aufgrund pathophysiologischer Gemeinsamkeiten zwischen Pferd und Mensch, so dass das Pferd ein anerkanntes Modelltier zur Erforschung und Behandlung orthopädischer Erkrankungen beim Menschen darstellt (PATTERSON-KANE et al. 2012).

In der vorliegenden Studie wurden von adulten Pferden Knochenmark, Fettgewebe und Sehnengewebe entnommen. Dabei standen gesunde Donorpferde zur Knochenmarkgewinnung zur Verfügung. Einige der Pferde wurden im Rahmen anderer Untersuchungen später euthanasiert, so dass unmittelbar post mortem eine zusätzliche Entnahme von Fett- und Sehnengewebe möglich war. Nabelschnurblut und Nabelschnurmaterial wurde während physiologischer Geburten gewonnen. Eine invasive Probenentnahme von Knochenmark, Fett- oder Sehnengewebe konnte an den jeweiligen Fohlen jedoch nicht durchgeführt werden. Eine Paarung der untersuchten Gewebeproben war damit nur teilweise möglich. Dies wurde in der statistischen Auswertung der Daten für den Vergleich der Zellquellen untereinander durch die Anwendung von Testverfahren für ungepaarte Proben (Kruskal-Wallis-Test und Mann-Whitney-U-Test) berücksichtigt.

Für die Knochenmarkgewinnung erfolgte eine Sternumpunktion nach allgemein üblichen Methoden am stehenden sedierten Pferd (ARNHOLD et al. 2007; BOURZAC et al. 2010). Die Entnahme war ohne Komplikationen durchführbar, obwohl in der Literatur Schwierigkeiten durch versehentliche Punktionen des Perikards beschrieben sind

(KASASHIMA et al. 2011). Weiterhin befindet sich der Tierarzt, bedingt durch den Entnahmeort am Brustbein, in einer gefährlichen Position bei Abwehrbewegungen des Pferdes (DELLING et al. 2012). Alternative Methoden beschreiben auch die Knochenmarkentnahme am Hüfthöcker, wie sie auch für andere Tierarten angewendet wird. Obwohl für Knochenmark-MSCs beider Entnahmeorte ähnliche Wachstumseigenschaften beschrieben sind, ist die zu erzielende Zellzahl am Hüfthöcker signifikant niedriger (DELLING et al. 2012). Aus diesem Grund wurde in der vorliegenden Studie die Knochenmarkgewinnung durch die übliche Sternumpunktion durchgeführt.

Subkutanes Fettgewebe wurde unmittelbar post mortem über eine Schnittinzision lateral der Schweifrübe entnommen; dies entspricht der beschriebenen Methode zur Entnahme von Fettgewebe am stehenden sedierten Pferd (RAABE et al. 2011; VIDAL et al. 2007). Komplikationen sind bei dieser Prozedur kaum zu erwarten, allerdings kann es aufgrund des Hautschnittes zu einer Narbenbildung im Entnahmebereich kommen. Dennoch handelt es sich hierbei um eine etablierte Methode der Fettgewebsgewinnung beim Pferd, mit der zuverlässig MSCs isoliert werden können.

Zur Gewinnung von Sehnengewebe wurde die oberflächliche Beugesehne (OBS) an frisch euthanasierten Pferden am palmaren Anteil des Röhrbeins verwendet. Eine Entnahme der OBS ist beim Patienten jedoch nicht praktikabel, da diese Sehne für den physiologischen Bewegungsablauf notwendig ist. Alternative Möglichkeiten der Gewinnung von MSCs aus Sehnengewebe bestehen jedoch in der Nutzung von weniger bedeutenden Sehnenstrukturen. Hier könnten sich zum Beispiel die Spatsehne (Musculus tibialis cranialis) oder die gemeinsame Strecksehne (Musculus extensor digitalis communis) als praktikabel erweisen. Für diese Studie wurde trotz der oben beschriebenen Einschränkungen auf die OBS als Zellquelle zurückgegriffen, da diese aufgrund der hohen Erkrankungshäufigkeit beim Pferd (DOWLING et al. 2000; KASASHIMA et al. 2004) oft Gegenstand von Untersuchungen ist und außerdem eine ausreichend große Gewebemenge verfügbar war. Eine andere Möglichkeit, dennoch MSCs aus der OBS zu isolieren, besteht in einem allogenen Einsatz von MSCs. In Studien wurde bereits die allogene Verwendung von MSCs untersucht, bei der keine vermehrten immunologischen Reaktionen nach Injektion von allogenen MSCs aus Plazenta oder Knochenmark beschrieben worden sind (CARRADE et al. 2011; GUEST et al. 2008). Eine genaue immunologische Charakterisierung von MSCs aus Sehnengewebe steht jedoch noch aus.

Nabelschnurblut und Nabelschnurmatrix wurden im Sächsischen Hauptgestüt Graditz und in der Ambulatorischen und Geburtshilflichen Tierklinik der Universität Leipzig gewonnen. Die Entnahme der entsprechenden Gewebe konnte nicht-invasiv durchgeführt werden. Es wurden keinerlei Komplikationen für Stute und Fohlen beobachtet, wie auch bereits in einer anderen Studie beschrieben worden war (BARTHOLOMEW et al. 2009). Zu beachten ist bei der Probengewinnung jedoch, dass in verschiedenen Studien zur Isolierung von MSCs aus Nabelschnurblut sowohl bei humanen als auch bei equinen Patienten nicht immer eine erfolgreiche Zellisolierung erzielt werden konnte (KERN et al. 2006; SCHUH et al. 2009). Weiterhin setzt die Gewinnung von Nabelschnurblut und Nabelschnurmaterial ein entsprechendes Management voraus, da das Gewebe direkt im Anschluss an die Geburt entnommen werden muss. Um dabei den natürlichen Geburtsablauf nicht zu stören, ist erfahrenes Personal vor Ort während der Geburt notwendig, das auch mit der Probenentnahme vertraut ist. Außerdem sollte beachtet werden, dass durch die typische Stallflora eine mögliche Kontamination der gewonnenen Proben erfolgen kann. Aus diesem Grund wurden in der hier vorliegenden Studie die Nabelschnurmaterialproben nach der Entnahme zunächst durch eine Waschung in Jodlösung und Alkohol desinfiziert und anschließend in einer Pufferlösung mit Antibiotika- und Antimykotikazusatz gelagert, bis eine weitere Verarbeitung im Labor stattfand. Auch während der Isolierung und der Anzüchtung der Nabelschnurgewebe-MSCs wurden für die Digestionslösung und für die Zellkulturmedien der Primärkultur Antibiotika und Antimykotika zugesetzt, wie bereits in verschiedenen Studien beschrieben (BARTHOLOMEW et al. 2009; LOVATI et al. 2011b; PASSERI et al. 2009). Dennoch konnte in der hier vorliegenden Studie bei der Anzüchtung von 2 aus 12 Nabelschnurmaterialproben eine bakterielle Kontamination oder eine Besiedelung mit Schimmelpilzen beobachtet werden, so dass diese Proben zur weiteren Kultivierung nicht mehr zur Verfügung standen. Daraus lässt sich schließen, dass die vorgenommenen Maßnahmen zur Erzielung steriler Proben nicht immer ausreichend waren und für weitere Versuche intensivere Protokolle zur Vermeidung von Kontaminationen angewendet werden sollten.

Die Isolierung der MSCs aus den verschiedenen Geweben wird durch unterschiedliche Verfahren erzielt. Dabei unterscheiden sich bereits die soliden Gewebe und Körperflüssigkeiten in der Isolationsmethode, bedingt durch die Konsistenz des Quellgewebes. Für Flüssigkeiten ist es dementsprechend ausreichend die lose vorkommenden Zellen aus der Lösung zu selektieren. In festen Geweben existieren die Zellen in einem

Gewebeverband, aus dem die Zellen herausgelöst werden müssen, unter anderem durch eine Zerstörung der Gewebegrundsubstanz.

In der vorliegenden Studie erfolgte die Isolierung von MSCs aus Knochenmark und Nabelschnurblut mittels eines Standardprotokolls über Dichtegradientenzentrifugation, wobei zunächst die mononukleären Zellen (MNCs) herausgefiltert werden und daraus nach weiterer Kultivierung MSCs aufgrund ihrer plastikadhärenten Eigenschaften gewonnen werden können. Eine Studie zu verschiedenen Isolationsprotokollen an equinen Knochenmark-MSCs zeigt dabei unterschiedlich erzielbare Zellzahlen sowie Veränderungen in der Zellviabilität (BOURZAC et al. 2010). Dies könnte dadurch bedingt sein, dass verschiedene Trennverfahren zu einer selektiven Isolierung verschiedenartiger Zellen führen, die mit unterschiedlichem Proliferationsverhalten der Zellen einhergehen. Da in den meisten Fällen die erhaltenen Zellen nur aufgrund ihrer phänotypischen Eigenschaften, unter anderem der spindelförmigen Form der Zellen, und aufgrund ihrer Plastikadhärenz zur weiteren Kultivierung genutzt werden, fällt eine genaue vergleichende Beschreibung der initial isolierten Zellen schwer. Um in der vorliegenden Studie gleiche Voraussetzungen für die Zellisolierung zu schaffen, wurde die Isolation von MSCs aus Knochenmark und Nabelschnurblut nach demselben etablierten Standardprotokoll mittels einer Auftrennung der Zellen aufgrund eines Dichtegradienten durchgeführt (ARNHOLD et al. 2007; VIDAL et al. 2012).

Deutlich anders gestaltet sich die Freisetzung von Zellen aus dem Gewebeverband in festen Geweben. Voraussetzung dafür ist eine mechanische oder chemische Zerstörung der Gewebestrukturen, die dann in einer passiven Herauslösung der Zellen aus den Gewebebestandteilen resultiert. Andererseits besteht die Möglichkeit, dass enthaltene Zellen aktiv den Gewebeverband verlassen und dadurch eine Isolierung der Zellen erfolgen kann.

Zur passiven Isolation von Zellen aus soliden Geweben durch eine Zerstörung des Gewebeverbandes wird häufig ein enzymatischer Gewebeverdau angewendet (COLLEONI et al. 2009; MENSING et al. 2011; PASSERI et al. 2009; RODBELL 1964; WAGENHAUSER et al. 2012). Hierfür existieren verschiedene Protokolle, die in den verwendeten Enzymen, ihren Kombinationen oder der Einwirkungsdauer der Enzymlösung variieren. Unabhängig davon, welches Digestionsprotokoll verwendet wird, bleibt es fraglich, inwiefern die Zellen durch die Digestionslösung beeinflusst werden. Neben einer Schädigung der MSCs durch enthaltene Toxine, entstandene Gewebezerfallsprodukte oder ein unphysiologisches Milieu während des Enzymverdaus kann auch eine Aktivierung oder Stimulierung der Zellen

während der Inkubation vermutet werden (BAPTISTA et al. 2009; FINK et al. 2011; HEFLEY et al. 1981; HYDER 2005; TSAGIAS et al. 2011; WILLIAMS et al. 1995).

Eine Alternative zur Isolierung von MSCs aus soliden Geweben ohne Einsatz von Enzymlösungen stellt die Explantationsmethode dar (LEE et al. 2011; SANCHEZ-GUIJO et al. 2009). Hierbei werden die gewonnenen Gewebestückchen zerkleinert auf einer adhärenten Zellkulturschale ausgebracht und mit Standardzellkulturmedium überschichtet, so dass die plastikadhärenten MSCs aktiv aus dem Gewebe auswandern und auf dem Boden der Zellkulturschale fest haften können. Dies ist auch für die Isolierung von Zellen aus Sehnengewebe beschrieben (WAGENHAUSER et al. 2012). Vorteilhaft könnte sich bei dieser Methode erweisen, dass die Zellen zunächst weiterhin in ihrem gewohnten Milieu existieren und dadurch nur eine minimale Beeinflussung des Zellcharakters während der Isolierung auftreten könnte. Fraglich ist jedoch, ob nur eine bestimmte Zellpopulation auswandert, wohingegen bei dem enzymatischen Verdau eventuell eine größere Mischpopulation isoliert wird. Deutliche Unterschiede dieser beiden Methoden sind vor allem in der isolierten Zellmenge zu erwarten (BAPTISTA et al. 2009; JAKOB et al. 2003; LEE et al. 2011; WAGENHAUSER et al. 2012). Die Gewinnung einer ausreichend großen Zellzahl stellt jedoch ein wichtiges Kriterium für einen praktikablen Einsatz der gewählten Isolationsmethode für die klinische Anwendung von MSCs dar (SEKIYA et al. 2002; YANG et al. 2011).

Aus diesem Grund wurde in der hier vorliegenden Studie die Zellausbeute der jeweiligen Proben anhand eines Standardverfahrens bestimmt, sowohl für die verschiedenen Gewebe als auch für die verschiedenen Isolationsmethoden. Dabei erfolgte eine lichtmikroskopische Zählung lebender Zellen mit Hilfe der Zählkammer nach Neubauer im Anschluss an eine Lebend-Tot-Färbung der Zellsuspension mit Trypanblau. Für Körperflüssigkeiten konnte dabei sowohl die MNC-Zahl bestimmt und verglichen werden als auch die MSC-Zahl nach Primärkultur. Ähnliches konnte für die soliden Gewebe durchgeführt werden, bei denen MSCs mittels der Digestionsmethode isoliert wurden. Hierbei war es möglich, die MNC-Zahl nach erfolgtem Gewebeverdau und die MSC-Zahl nach anschließender Kultivierung am Ende der Passage 0 zu bestimmen. Für die Explantationsmethode konnte nur die MSC-Zahl nach Primärkultur ermittelt werden, da eine Bestimmung der tatsächlichen Anzahl an ausgewanderten Zellen nicht möglich war.

Die Beurteilung der Eignung der durchgeführten Isolation von MSCs aus verschiedenen Geweben zur routinemäßigen Anwendung sollte jedoch nicht nur anhand initialer Zellzahlen

erfolgen. Wichtig ist auch, vor allem für klinische Applikationen von MSCs, nach Kultivierung ausreichend große Zellzahlen zur erzielen (JAKOB et al. 2003; LEE et al. 2011). Dafür ist eine adäquate und erfolgreiche Vermehrung der Zellen im Labor notwendig. Eine Aussage zum Proliferationsverhalten von MSCs kann über die Bestimmung der Generationszeiten erfolgen, wie in diesen Studien nach Standardmethoden über Bestimmung von Zellzahlvermehrung, Kultivierungs- und Generationszeiten bis zur Passage 7 durchgeführt wurde (VIDAL et al. 2006). Zusätzlich erfolgte zur Einschätzung des Proliferationsverhaltens ein modifizierter 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazoliumbromid (MTT)-Assay in Passage 3 und Passage 8. Dabei wurde mittels photometrischer Messungen des gebildeten Formazanproduktes eine Aussage über die Anzahl viabler Zellen getroffen.

Neben dem Proliferations- und Wachstumspotential ist für eine spätere klinische Anwendung auch das Migrationspotential der kultivierten MSCs bedeutsam. Gute Migrationseigenschaften könnten sich dabei für eine optimale Integration der applizierten MSCs in das erkrankte Gewebe als vorteilhaft erweisen. Um dies in vitro zu untersuchen wurde in der vorliegenden Studie zunächst eine Kultivierung der Zellen in einem dreidimensionalen System durchgeführt. Dieses Sphäroidsystem ähnelt eher den Bedingungen in vivo und könnte sich außerdem für eine lokale Applikation der MSCs bei klinischer Anwendung als vorteilhaft erweisen. Die in-vitro-Migration der Zellen wurde dann anhand der Auswanderungsfähigkeit der MSCs aus dem verwendeten Sphäroidsystem mikroskopisch untersucht und dokumentiert. Als Parameter zur Beurteilung des Migrationspotentials wurde dabei die Fläche auf der Zellkulturschale um die MSC-Kultur bestimmt, die in einer definierten Zeitspanne von ausgewanderten MSCs besiedelt worden war. Solche in-vitro-Untersuchungen geben erste Hinweise auf das Verhalten der MSCs, der tatsächliche Effekt im lebenden Organismus sollte jedoch durch umfangreiche in-vivo-Studien gestützt werden.

Gleiches gilt für die Beurteilung des Differenzierungsverhaltens der erhaltenen MSCs in vitro. Zum einen ist dadurch der Nachweis ihrer charakteristischen Multipotenz möglich (DOMINICI et al. 2006), die in der vorliegenden Studie anhand der adipogenen, osteogenen und chondrogenen Differenzierung von MSCs in Passage 3 durchgeführt wurde, zum anderen lassen sich möglicherweise erste Rückschlüsse hinsichtlich einer selektiven Anwendung bestimmter MSCs für spezifische Erkrankungen erkennen.

Die osteogene und chondrogene Differenzierung sowie der histologische Nachweis der erfolgreichen Differenzierung der MSCs wurden in dieser Studie nach Standardprotokollen durchgeführt (ARNHOLD et al. 2007; GIOVANNINI et al. 2008; TOUPADAKIS et al. 2010;

VIDAL et al. 2006). Für die adipogene Differenzierung wurde ein modifiziertes Protokoll mit Zusatz von Kaninchenserum angewendet (JANDEROVA et al. 2003). Wie eigene Voruntersuchungen und auch die vorliegende Studie bestätigten, konnte hierbei eine verlässliche Differenzierung der MSCs aus allen equinen Zellquellen ermöglicht werden, wohingegen in anderen Studien für equine MSCs teilweise eine unzureichende oder nicht erfolgreiche adipogene Differenzierung nach Standardmethoden beschrieben wurde (GITTEL et al. 2011; MENSING et al. 2011; RANERA et al. 2012).

Eine Evaluierung der Differenzierungsfähigkeit erfolgte anschließend anhand histologischer Färbungen, die für adipogen und chondrogen differenzierte MSCs semiquantitativ mittels eines Punktesystems ausgewertet wurden. Dabei wurde im Falle der adipogenen Differenzierung eine Schätzung des Anteils der differenzierten Zellen sowie eine Beurteilung der entwickelten Lipidtropfen durchgeführt (GITTEL et al. 2011). Für die Bewertung der chondrogenen Differenzierung der MSCs wurde der „BernScore“ (GROGAN et al. 2006) angewendet, bei dem neben der Intensität der Safranin-O-Färbung zusätzlich Zellabstand, Matrixmenge und Zellmorphologie beurteilt wurden. Damit konnten chondrogene Zellpellets unterschiedlicher Reifestadien semiquantitativ eingestuft werden (OTSUKI et al. 2010).

Osteogen induzierte MSCs wurden neben einem qualitativen Nachweis mit Hilfe der von Kossa Färbung auch anschließend quantitativ durch photometrische Messungen verglichen, wie bereits in der Literatur beschrieben (OSTANIN et al. 2008). Als Negativkontrollen fanden dafür nicht induzierte MSCs in Monolayerkultur Verwendung.

Im Hinblick auf eine spätere klinische Nutzung bei Sehnenerkrankungen wurde in der hier vorliegenden Studie die Genexpression mittels Real-Time-Polymerase-Kettenreaktion (PCR) untersucht. Ziel dabei war es festzustellen, ob die Isolationsmethode oder die Zellquelle Einfluss auf die Genexpression der MSCs haben. Zielgene waren die Sehnenmarker Kollagen 1A2 sowie Skleraxis, die in Sehnengewebe auf sehr hohem Level exprimiert werden (ASLAN et al. 2008; SALINGCARNBORIBOON et al. 2003; SHARMA und SNEDEKER 2010; TAYLOR et al. 2009; THORPE et al. 2010; WAGENHAUSER et al. 2012; WANG et al. 2005). Damit könnten diese Marker ein potentielles Kriterium für die Eignung von bestimmten MSCs zur klinischen Anwendung bei Sehnenerkrankungen beim Pferd darstellen. Zu beachten ist jedoch, dass damit nur die Basisexpression auf Transkriptionsebene untersucht wurde und keinerlei Aussage über die tatsächliche Translation gemacht werden kann. Hierzu sind weiterführende Untersuchungen notwendig.

Neben der Fähigkeit der Multipotenz wurden in der hier vorliegenden Studie die isolierten Zellen zusätzlich anhand der Selbstreplikation und Plastikadhärenz als MSCs identifiziert. Diese Parameter gelten als minimale, aber ausreichende Kriterien zur Identifikation von equinen MSCs (BI et al. 2007; BOURZAC et al. 2010; MENSING et al. 2011). Sicher wäre eine zusätzliche Beurteilung der Expression von Oberflächenmarkern exakter. Allerdings ergeben sich hierbei für Pferdezellen einige Schwierigkeiten durch eine limitierte Kreuzreaktivität der verfügbaren Antikörper mit den equinen Zellen. Einige Studien beschäftigen sich mit vielversprechenden Ergebnissen mit dieser Problematik, dennoch existiert für equine MSCs derzeit noch keine Definition eines standardmäßigen Sets an Oberflächenmarkern, wie es für humane Zellen bereits etabliert ist (DE SCHAUWER et al. 2012; IBRAHIM und STEINBACH 2012). Weiterführende Studien zur eindeutigen Charakterisierung equiner MSCs sind deshalb notwendig.

5.2 Diskussion der Ergebnisse

In der hier vorliegenden Studie wurde das Proliferations-, Migrations- und Differenzierungsverhalten sowie das Genexpressionsmuster von Sehnenmarkern in MSCs aus verschiedenen equinen Geweben untersucht. Dabei standen zum einen Knochenmark und Nabelschnurblut als Zellquellen zur Verfügung, aus denen aus allen verwendeten Proben erfolgreich MSCs isoliert werden konnten. Damit ist die Erfolgsquote zur Zellisolierung in dieser Studie höher als zum Teil in der Literatur für humanes oder equines Nabelschnurblut beschrieben wurde (BARTHOLOMEW et al. 2009; KERN et al. 2006). Zum anderen wurden aus soliden equinen Geweben (Fettgewebe, Sehnengewebe und Nabelschnurmateria)l erfolgreich MSCs isoliert, sowohl mittels Digestion als auch mittels Explantation.

Alle Proben an lebenden Tieren (Knochenmark, Nabelschnurblut, Nabelschnurmaterial) konnten ohne Komplikationen für das Spendertier entnommen werden. In der Literatur sind jedoch auch ernsthafte Zwischenfälle, bis hin zum Tod nach der invasiven Knochenmarkentnahme am Sternum beschrieben, die aufgrund von versehentlichen Punktionen des Perikards entstehen können (DELLING et al. 2012; KASASHIMA et al. 2011). Weiterer Nachteil dieser Entnahmetechnik ist das Risiko für den punktierenden Tierarzt aufgrund von Abwehrbewegungen des Pferdes, da er sich in einer ungünstigen Position nahezu unter dem Pferd befindet. Weniger risikobehaftet ist daher die Gewinnung von geburtsassoziierten Geweben. Die hier gemachten Beobachtungen zu Komplikationen für

Stute und Fohlen entsprechen denen einer anderen Studie (BARTHOLOMEW et al. 2009). Aufgrund dieser komplikationslosen Entnahmemöglichkeit stellen diese Gewebe eine gute Alternative zu Knochenmark da.

Fettgewebe wurde in der vorliegenden Studie post mortem entnommen. Eine Gewinnung am lebenden Tier ist jedoch ohne größere Komplikationen möglich und wird auch in einigen Studien beschrieben (RAABE et al. 2011; VIDAL et al. 2012).

Die hier durchgeführte Entnahme der OBS zur Gewinnung von Sehnengewebe ist am lebenden Tier nicht möglich, da die OBS eine entscheidende Bedeutung im natürlichen Bewegungsablauf des Pferdes hat. Im Hinblick auf eine eventuelle allogene Nutzung von MSCs kann jedoch eine solche Entnahme durchgeführt werden. Wichtig ist dabei jedoch, dass vitale Zellen auch bei post mortem entnommenen Geweben isoliert werden können. Dies war in der vorliegenden Studie für alle gewonnenen Proben möglich.

Zusammenfassend lässt sich damit sagen, dass aus allen untersuchten Gewebeproben erfolgreich MSC isoliert werden konnten, die damit einer weiteren Expansion und Proliferation zugeführt werden konnten. Auffallend während der Anzüchtung der verschiedenen Zellen war, dass Kulturen aus Nabelschnurmaterial zum Teil ein Auftreten von bakteriellen Kontaminationen oder einen Schimmelpilzbefall zeigten, obwohl ein Zusatz von Antibiotika und Antimykotika in allen verwendeten Lösungen vorlag. Anscheinend erwies sich dies jedoch als unzureichend, so dass für weiterführende Studien eine höhere, längere oder alternative Supplementierung von Medien mit Antibiotika und Antimykotika erfolgen sollte.

Hinsichtlich einer späteren therapeutischen Anwendung von MSCs ist jedoch nicht nur die erfolgreiche Anzüchtung der MSCs, sondern auch die Gewinnung einer ausreichend großen Anzahl vitaler Zellen bedeutsam (JAKOB et al. 2003; LEE et al. 2011; SMITH 2008). Die Ergebnisse der hier vorliegenden Studie zeigen, dass aus den untersuchten Körperflüssigkeiten 222-fach weniger MSCs im Vergleich zu soliden Geweben isoliert werden konnten. Allgemein glichen die Zellzahldimensionen jedoch den in der Literatur beschriebenen Angaben zur Isolierung von MSCs aus verschiedenen Quellen (LEE et al. 2011; YANG et al. 2011).

Hinsichtlich des Isolationsprotokolls konnten bei den soliden Geweben im Vergleich zur Explantationsmethode mittels enzymatischem Verdau signifikant mehr MSCs isoliert werden, wie auch schon in verschiedenen Studien beschrieben wurde (BAPTISTA et al. 2009; JAKOB

et al. 2003; LEE et al. 2011). Mögliche Ursache hierfür könnte sein, dass während des Explantationsverfahrens nur die Zellen, die am Geweberand sitzen, aus dem Gewebestück auswandern und an der Zellkulturschale anhaften können. Weiterhin besteht bei dem Gewebeverdau die Möglichkeit, dass eine größere Mischpopulation von Zellen gewonnen wird, wodurch die initialen Zellerträge auch höher sein könnten (WAGENHAUSER et al. 2012).

Im Hinblick auf das Proliferationspotential war auffallend, dass MSCs aus Nabelschnurblut und aus Knochenmark deutlich mehr Kultivierungszeit zur Vermehrung benötigten und damit längere Generationszeiten sowie Seneszenz aufwiesen. Dies ist im Einklang mit anderen Studien über equine und humane MSCs, die für Knochenmark-MSCs ein niedrigeres Proliferationspotential und eine hohe Seneszenz nachwiesen im Vergleich mit MSCs aus anderen Geweben (KERN et al. 2006; VIDAL et al. 2012). Allerdings wurde in einer der Studien gezeigt, dass humane MSCs aus Nabelschnurblut ein sehr gutes Proliferationsverhalten besitzen, wenn sie nicht bereits in frühen Passagen seneszent geworden waren (KERN et al. 2006).

Als weiteres Zellcharakteristikum wurde in der vorliegenden Studie das Migrationsverhalten nach einer dreidimensionialen Kultivierung der MSCs untersucht. Dabei zeigte sich eine deutlich langsamere Migration von MSCs aus Knochenmark und Nabelschnurmaterial. Ähnliche Rückschlüsse lassen sich auch aus einer Studie ziehen, bei der MSCs aus Knochenmark nach intraläsionaler Applikation in geschädigte Beugesehnen nahe am Applikationsort verbleiben (GUEST et al. 2008). Weiterhin könnte die Isolationsmethode die MSCs in ihrem Migrationspotential beeinflussen; dazu konnten in der vorliegenden Studie aber keine signifikanten Unterschiede aufgezeigt werden. Jedoch war eine Tendenz zu erkennen, dass MSCs aus soliden Geweben, die mittels enzymatischem Verdau isoliert wurden, eine schnellere Migration zeigen. Dies könnte damit erklärt werden, dass die Gegebenheiten der Isolationsprozedur das Migrationsverhalten beeinflussen, wie auch bereits für verschiedene Zusätze in der Zellkultivierung beschrieben ist (KIM et al. 2010; MULLER et al. 2011; TSAI et al. 2006). Entgegen den hier gestellten Erwartungen zeigten die mittels Explantation isolierten MSCs kein besseres Migrationsverhalten, obwohl bei dieser Methode eine eventuelle selektive Isolierung und Anzüchtung von MSCs mit guter Migrationsfähigkeit vorgelegen haben könnte.

Die charakteristische Multipotenz von MSCs wurde in der vorliegenden Studie durch die adipogene, osteogene und chondrogene Differenzierung der isolierten Zellen in vitro nachgewiesen.

Für die Induktion der adipogenen Differenzierung wurde hierbei ein modifiziertes Protokoll mit Zusatz von Kaninchenserum erfolgreich angewendet. Kein Unterschied der adipogenen Differenzierungspotenz konnte sowohl zwischen den MSCs aus verschiedenen Quellen als auch bei den unterschiedlich isolierten Zellen gefunden werden. Dies spricht dafür, dass sich die hier untersuchten MSCs nach erfolgreicher Induktion gleich gut adipogen differenzieren lassen.

Unterschiede der Differenzierungsfähigkeit zwischen den MSCs aus verschiedenen Quellen konnten jedoch im Hinblick auf die osteogene und chondrogene Differenzierung beobachtet werden. Dabei zeigten MSCs aus Knochenmark ein sehr gutes osteogenes Differenzierungspotential, wobei sich dieser Unterschied mit zunehmender Inkubationszeit verstärkte. Auch andere Autoren berichten über die sehr gute osteogene Differenzierungsfähigkeit von MSCs aus Knochenmark in vitro (TOUPADAKIS et al. 2010). Ebenfalls in Übereinstimmung mit der Literatur steht die Beobachtung der schlechteren osteogenen Differenzierungsfähigkeit von MSCs aus Nabelschnurblut und Nabelschnurgewebe (HOYNOWSKI et al. 2007; KOCH et al. 2007; PASSERI et al. 2009; REED und JOHNSON 2008).

Hinsichtlich der chondrogenen Differenzierungsfähigkeit konnte das gute Differenzierungspotential von MSCs aus Nabelschnurblut, dass in einer weiteren Studie bereits nachgewiesen wurde, auch in der hier durchgeführten Untersuchung bestätigt werden (BERG et al. 2009). Andererseits widerspricht die in der vorliegenden Studie beobachtete mäßige chondrogene Differenzierung von Knochenmark-MSCs den Ergebnissen anderer Studien (GIOVANNINI et al. 2008; LOVATI et al. 2011b). Eine Erklärung für diesen Unterschied könnte in einer unterschiedlichen Heterogenität der isolierten Zellpoplulationen oder aber in interindividuellen Unterschieden der Donortiere liegen, wie in einer anderen Studie an equinen MSCs vermutet worden war (CARTER-ARNOLD et al. 2013).

Im Gegensatz zu dem unterschiedlichen Differenzierungspotential von MSCs aus verschiedenen Quellen zeigten sich im Hinblick auf die Isolationsmethode keine signifikanten Unterschiede im Differenzierungspotential bei MSCs aus soliden Geweben. Dies entspricht den Ergebnissen anderer Studien, bei denen die Auswirkungen verschiedener Isolations-

protokolle untersucht wurden (BOURZAC et al. 2010; LEE et al. 2011). Damit scheint der Einfluss extrinsischer Faktoren, wie zum Beispiel der Isolationsmethode, unbedeutender zu sein, sobald eine erfolgreiche Induktion der Differenzierung stattgefunden hat. Mögliche Unterschiede aufgrund von verschiedenen Kulturbedingungen wurden in der hier vorliegenden Studie vermieden, indem nach erfolgter Isolierung die Kultivierung und Differenzierung der MSCs nach optimierten und standardisierten Methoden durchgeführt wurde.

Somit lässt sich schlussfolgern, dass vor allem die Herkunft der MSCs einen maßgeblichen Einfluss auf das Differenzierungspotential hat. Inwiefern sich dieser Unterschied auch auf in-vivo-Anwendungen auswirken kann, ist nicht eindeutig geklärt.

Im Hinblick auf die Genexpression der Sehnenmarker Kollagen 1A2 und Skleraxis konnten deutliche Unterschiede zwischen den MSCs aus verschiedenen Quellen gefunden werden. Beide Gene sind als Marker für Sehnengewebe oder Sehnenentwicklung in verschiedenen Publikationen untersucht worden (ASLAN et al. 2008; SALINGCARNBORIBOON et al. 2003; SHARMA und SNEDEKER 2010; TAYLOR et al. 2009; THORPE et al. 2010; WAGENHAUSER et al. 2012; WANG et al. 2005). In der hier vorliegenden Studie zeigten MSCs aus Fettgewebe eine deutlich höhere Kollagenexpression und MSCs aus Nabelschnurblut eine deutlich höhere Skleraxisexpression als MSCs aus anderen Quellen, was durch eine eventuelle Vordifferenzierung der MSCs abhängig vom Ursprungsgewebe bedingt sein könnte. MSCs aus Sehnengewebe exprimierten beide Sehnenmarker auf relativ hohem Level. Dies könnte für einen potentiellen Vorteil dieser MSCs im klinischen Einsatz bei Sehnenerkrankungen sprechen. Hinsichtlich der Isolationsmethode könnten sich bei MSCs aus soliden Geweben die mittels Digestion isolierten Zellen als vorteilhaft erweisen, die im Vergleich mit den mittels Explantation isolierten MSCs eine höhere Expression von Skleraxis aufwiesen. Dies könnte durch eine Hochregulation von Skleraxis aufgrund von Kollagenabbauprodukten während des enzymatischen Verdaus bedingt sein.

Da ähnliche eindeutige Differenzen zwischen den unterschiedlich isolierten MSCs für Kollagen 1A2 nicht beobachtet werden konnte, spricht das für ein stabileres Expressionslevel von Kollagen 1A2. Dies wurde bereits in einer Studie über den Einfluss von Antiphlogistika auf die Kollagenexpression geschlussfolgert (TSAI et al. 2010). Allerdings konnten für Fettgewebs- und Sehnengewebs-MSCs tendenziell höhere Kollagenexpressionslevel bei den mittels Digestion isolierten MSCs gefunden werden. Ähnliches wurde in einer Studie mit enzymatisch isolierten und anschließend kultivierten humanen Tenozyten beobachtet

(WAGENHAUSER et al. 2012). Inwiefern diese Unterschiede in der Genexpression bei den verschiedenen Isolationsmethoden allerdings durch eine Isolierung verschiedener Zellpopulationen verursacht sein könnten, bleibt fraglich.

Eine vergleichende Untersuchung der tenogenen Differenzierungsfähigkeit könnte eindeutigere Aussagen bezüglich einer optimierten klinischen Anwendung der MSCs treffen, aber aufgrund fehlender Standardprotokolle sowie nur mangelhafter Möglichkeiten für den exakten Nachweis der tenogenen Differenzierung wurde hier die Analyse der Basisexpression von Sehnenmarkern vorgezogen.

5.3 Ausblicke auf die Relevanz für die klinische Anwendung von MSCs

Zusammenfassend lässt sich sagen, dass MSCs aus allen untersuchten Geweben erfolgreich isoliert werden konnten. Die Gewebeentnahmen waren in allen Fällen ohne Komplikationen möglich, obwohl bei der invasiven Entnahmetechnik für Knochenmark ernsthafte Zwischenfälle für die Donorpferde beschrieben worden sind (KASASHIMA et al. 2011). Die mögliche Narbenbildung nach Fettgewebsentnahme konnte in der vorliegenden Studie nicht weiter verfolgt werden, da hier eine Gewinnung an frisch euthanasierten Pferden stattgefunden hat. Größere Komplikationen am lebenden Tier sind aber nicht zu erwarten. Für die Gewinnung von Sehnengewebe ist die autologe Nutzung von OBS-Material nicht angebracht, alternative Sehnenstrukturen oder eine allogene Gewinnung von Spendertieren stellen jedoch eine gute Möglichkeit der MSC-Gewinnung aus diesem Gewebe dar. Die geburtassoziierten Gewebe Nabelschnurblut und Nabelschnurmaterial sind in der Humanmedizin als mögliche MSC-Quellen verbreitet (KERN et al. 2006). Auch für Pferde erscheinen diese Gewebe potentiell nutzbar, jedoch ist hier ein entsprechendes Entnahmemanagement und Hygieneregime notwendig (SCHUH et al. 2009).

Im Vergleich der Gewebe untereinander zeigten sich MSCs aus Fett- und Sehnengewebe mit guten Proliferationseigenschaften und einer hohen erreichbaren Zellzahl und könnten damit in der Pferdemedizin eine gute Alternative zum routinemäßig verwendeten Knochenmark darstellen. Aufgrund der hohen Expression der untersuchten Sehnenmarker scheint Fett- und Sehnengewebe vor allem im Hinblick auf die Anwendung bei Sehnenerkrankungen potentiell vorteilhaft zu sein. Andere Indikationen könnten allerding andere Zellquellen benötigen, um

eine optimale Nutzung der spezifischen Eigenschaften der MSCs zu ermöglichen. Weiterführende in-vivo-Studien stehen hierzu jedoch noch aus.

Bei den soliden Geweben, die sich im Allgemeinen als vielversprechende Quellen für die MSC-Gewinnung zeigten, hat die Digestionsmethode Vorteile aufgrund der möglichen Isolierung höherer Zellzahlen im Vergleich zur Explantationsmethode. Dies ist als vorteilhaft für die in-vitro-Expansion einzustufen, da zur klinischen Anwendung von MSCs ausreichend Zellen nach möglichst kurzer Kultivierungszeit vorhanden sein sollten. Da in der vorliegenden Studie keine negativen Einflüsse des enzymatischen Gewebeverdaus auf die Zellqualität beobachtet werden konnte, kann diese Methode zur routinemäßigen Gewinnung von MSCs aus soliden Geweben empfohlen werden.

Vor allem im Hinblick auf die Anwendung bei Sehnenerkrankungen beim Pferd könnten sich MSCs, die mittels des Digestionsverfahrens isoliert wurden, als vorteilhaft erweisen. Diese Vermutung resultiert auch aus der in vitro vorliegenden höheren Expression des Sehnenmarkers Skleraxis im Vergleich mit MSCs, die mittels der Explantationsmethode isoliert wurden. Eine gesicherte Übertragbarkeit dieses Ergebnisses auf die Umstände im lebenden Tier steht jedoch noch aus.

Inwiefern die gefundenen Unterschiede in vitro zwischen den MSCs aus verschiedenen Quellen und den verschiedenen Isolationsmethoden auch bei klinischer Anwendung von MSCs relevant sind, sollte in weiterführenden in-vivo-Studien untersucht werden.

6 Zusammenfassung

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Einfluss von Ursprungsquelle und Isolationsmethode auf zellbiologische Charakteristika equiner mesenchymaler Stromazellen

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Multipotente mesenchymale Stromazellen (MSCs) stellen nicht nur beim humanen Patienten, sondern auch in der Veterinärmedizin einen vielversprechenden Therapieansatz in der Behandlung erkrankter muskuloskelettaler Gewebe dar. Ziel der Behandlung ist dabei die Regeneration der betroffenen Strukturen im Vergleich zur Reparation nach konservativer Therapie. Vor allem im Bereich von Sehnenerkrankungen können nach MSC-Applikation vielversprechende Ergebnisse im Hinblick auf niedrigere Rezidivraten beobachtet werden. Dennoch sind noch nicht alle Umstände einer optimalen MSC-Anwendung geklärt.

Hierbei sind unter anderem Fragen bezüglich der Herkunft und Gewinnung von MSCs offen, da Unterschiede von MSCs aufgrund ihrer Gewebezugehörigkeit bereits nachgewiesen wurden. Grundlegende umfassende Arbeiten zum Vergleich von equinen MSCs aus verschiedenen Quellen sowie deren mögliche Beeinflussung durch die Isolierung aus dem Gewebe lagen bislang noch nicht vor.

Ziel dieser Studie war es daher, equine MSCs aus verschiedenen Quellen zu gewinnen und mögliche Unterschiede in vitro aufzuzeigen. Weiterhin sollten Unterschiede zwischen den Zelleigenschaften nach Anwendung verschiedener Isolationsprotokolle untersucht werden.

In der hier vorliegenden Studie wurden MSCs aus Fett- und Sehnengewebe, Knochenmark, Nabelschnurblut und Nabelschnurgewebe von Pferden isoliert und vergleichend charakterisiert. Dabei wurden für die soliden Körpergewebe zwei unterschiedliche Isolationsmethoden, die Digestion und die Explantation, angewendet, um mögliche Einflüsse auf die gewonnen Zellen zu ermitteln.

Die untersuchten Kriterien beinhalteten Zellertrag, Proliferation, Differenzierungspotenz und das Migrationsverhalten von MSCs. Hinblickend auf eine Anwendung von MSCs bei Sehnenerkrankungen wurde auch die Expression von Sehnenmarkern verglichen.

In der vorliegenden Studie konnte gezeigt werden, dass sich die MSCs aus verschiedenen Quellen hinsichtlich der Zellausbeute und ihres Wachstumspotentials unterschieden. Aus soliden Geweben konnten mittels Digestion im Vergleich zu Körperflüssigkeiten signifikant mehr MSCs isoliert werden ($p < 0,001$). Dabei erbrachte die Isolation von MSCs mittels Digestionsmethode einen deutlich höheren Zellertrag nach der Passage 0 im Vergleich zur Explantationsmethode ($p < 0,05$). Im weiteren Verlauf der Kultivierung zeigten MSCs aus Sehnengewebe und Fettgewebe ein signifikant besseres Proliferationsverhalten im Vergleich zu Knochenmark-MSCs und Nabelschnurblut-MSCs.

Im Hinblick auf das Differenzierungspotential konnten signifikante Unterschiede zwischen den MSCs aus den verschiedenen Quellen beobachtet werden. MSCs aus Knochenmark zeigten eine sehr gute osteogene Differenzierungsfähigkeit im Vergleich zu MSCs aus den geburtsassoziierten Geweben ($p < 0,05$). Im Gegensatz dazu zeichneten sich diese MSCs durch eine deutlich bessere chondrogene Differenzierung im Vergleich zu Knochenmark-MSCs aus ($p < 0,05$). Im Hinblick auf die Isolationsmethode konnten keine Unterschiede im Differenzierungspotential beobachtet werden.

Weitere Unterschiede aufgrund der Zellquelle lassen sich in der Genexpression der Sehnenmarker erkennen. MSCs aus Fettgewebe und Sehnengewebe exprimierten Kollagen 1A2 auf höchstem Niveau. Sklexaris hingegen wurde von MSCs aus Nabelschnurblut und Sehnengewebe am höchstem exprimiert. Dabei zeigten MSCs, die mittels Digestionsmethode isoliert worden waren, ein signifikant höheres Expressionslevel von Skleraxis im Vergleich zur Explantationsmethode ($p < 0,05$).

Die Ergebnisse der vorliegenden Studie lassen einen Einfluss der Zellquelle auf die Zellcharakteristika erkennen. MSCs aus Fettgewebe stellen dabei eine vielversprechende Alternative zu Knochenmark-MSCs dar. Allerdings scheint für eine klinische Anwendung von MSCs eine selektive Auswahl der Zellquelle entsprechend der vorliegenden Erkrankung von Vorteil zu sein. Dabei ist eine Isolierung von MSCs aus soliden Geweben mittels Digestionsverfahren zu empfehlen, da hier deutlich höhere Zellzahlen gewonnen werden können. Eine negative Beeinflussung der Zelleigenschaften durch die enzymatische Digestion lässt sich nach den vorliegenden Ergebnissen nicht vermuten. Inwiefern die beobachteten Unterschiede bei in-vivo-Anwendungen von Bedeutung sind, muss jedoch noch umfassend untersucht werden.

7 Summary

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Influence of origin and isolation method on cell biological features of equine mesenchymal stromal cells

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Key words: horse, mesenchymal stromal cell, bone marrow, tendon tissue, umbilical cord, adipose tissue, isolation

Not only in humans but also in veterinary medicine, multipotent mesenchymal stromal cells (MSCs) are a promising treatment option in the therapy of injured musculoskeletal tissues. This is due to the improved tissue regeneration instead of the insufficient reparation following conventional therapies. With regard to an application of MSCs for treatment of tendinopathies in horses, lower rates of reinjury have been reported. However, further investigations to optimize the MSC treatment are still outstanding.

Differences in MSCs from different origins have been already reported, but there are still remaining questions about the influence of origin and isolation procedures of MSCs. Fundamental research on equine MSCs derived from different sources and their potential impact due to the isolation process has not been published so far.

The aim of this study was to isolate equine MSCs from different sources and to demonstrate potential differences in vitro. Furthermore, differences in cell features following different isolation methods were investigated. In the present study, MSCs from horses were isolated from adipose tissue, tendon tissue, bone marrow, umbilical cord blood and umbilical cord tissue and subsequently subjected to comparative characterization. In case of the solid tissues, two different isolation methods, digestion and explantation, were performed in order to analyze influences on obtained cells.

Investigated cell features included cell yield, proliferation, differentiation and migration potential. Furthermore, expression of tendon markers was evaluated with regard to an application of MSCs in tendinopathies.

In the present study it was shown that MSCs derived from different sources differ distinctly in cell yield and proliferation potential. In comparison to body fluids, significantly more MSCs could be isolated from solid tissues when using the digestion method ($p < 0.001$). Furthermore, the cell yield at first cell harvest was distinctly higher when performing the isolation by digestion in comparison to isolation by explantation ($p < 0.05$). With regard to further cultivation, MSCs derived from tendon tissue and adipose tissue displayed a significantly better proliferation potential compared to MSCs derived from other sources.

Considering the differentiation potential, significant differences were obvious between the MSCs derived from different sources. Bone marrow-MSCs showed an excellent osteogenic differentiation capacity in comparison to MSCs derived from umbilical cord blood and tissue ($p < 0.05$). In contrast, the birth-associated MSCs displayed a distinctly better chondrogenic differentiation than MSCs derived from bone marrow ($p < 0.05$). No difference in the differentiation potential was noticeable following the different isolation procedures.

Furthermore, differences in the gene expression of tendon markers were evident with regard to the cell source. MSCs derived from adipose tissue and tendon tissue expressed collagen 1A2 on the highest level. On the other hand, scleraxis was expressed highest in MSCs derived from umbilical cord blood and tendon tissue. In these cells, MSCs isolated by the digestion method showed a significantly higher expression level of scleraxis in comparison to MSCs isolated by explantation ($p < 0.05$).

Based on the results obtained so far, a relevant impact of the source of MSCs on cell features was evident. MSCs derived from adipose tissue are a promising alternative to bone marrow-MSCs. However, with regard to a clinical application of MSCs, a selection of the MSC source depending on the respective intended use seems to be advantageous. For routine isolation of MSCs from solid tissues, the digestion method could be recommended due to the higher obtainable cell numbers. Furthermore, a negative influence of the enzymatic digestion on the cell features was not detectable. However, to what extent the observed differences in vitro are relevant for in-vivo-applications needs to be further investigated.

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