TOWARDS A UNIFORM CHARACTERIZATION OF EQUINE MESENCHYMAL STROMAL CELLS

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Catharina DE SCHAUWER

Faculty of Veterinary Medicine

Department of Reproduction, Obstetrics and Herd Health

Salisburylaan 133 – 9820 Merelbeke - Belgium

Promotor: Prof. Dr. A. Van Soom

Co-Promotor: Prof. Dr. E. Meyer

Co-Promotor: Prof. Dr. G.R. Van de Walle

Een woordje uitleg bij de cover:

Stamcellen zijn bekend bij het grote publiek door de vele krantenartikels die regelmatig verschijnen. Jammer genoeg wordt er ook vaak verkeerde informatie gegeven, zowel uit onwetendheid als uit puur winstbejag. Het is dan ook de taak van de universiteit om niet alleen te waken over het publiceren van correcte informatie maar zeker ook over haar wetenschappelijke integriteit.

Om het met de woorden van Michael Evans te zeggen: “If you don’t stand for something, you’ll fall for anything”.

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I'd rather lose myself in passion than lose my passion

Jacques Mayol

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LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ASC	adult stem cells
AT	adipose tissue
BM	bone marrow
BSA	bovine serum albumin
CD	cluster of differentiation
CDN	cell-doubling number
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
ESC	embryonic stem cells
FCS	fetal calf serum
HBSS	Hank's Balanced Salt Solution
HES	hydroxyethyl starch
HR	hazard ratio
IFN- γ	interferon-γ
ISCT	International Society of Cellular Therapy
IQR	interquartile range
mAbs	monoclonal antibodies
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MNC	mononuclear cells
MSC	mesenchymal stem / stromal cells
NH_4Cl	ammonium chloride
PB	peripheral blood
PBS	phosphate buffered saline
PDT	population-doubling time
PI	propidium iodide
RBC	red blood cells

RT	room temperature
SEM	standard error of the mean
UCB	umbilical cord blood
UCM	umbilical cord matrix

CHAPTER 1

GENERAL INTRODUCTION

Adapted from: De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011;75:1431-43.

Adapted from: De Schauwer C, Van de Walle GR, Meyer E, Van Soom A. Mesenchymal stem cell therapy in horses: useful beyond orthopedic injuries? Stem Cells and Development, submitted.

1. Stem cells

1.1. General definition and classification

A stem cell is defined by three criteria: ability of (i) self-renewal, (ii) differentiation into multiple cell types, and (iii) *in vivo* reconstitution of a given tissue (Lakshmipathy & Verfaillie, 2005). Self-renewal implies that a cell can undergo either symmetric divisions into daughter cells which retain full stem cell characteristics, as such maintaining the stem cell population, or asymmetric divisions where only one of the two daughter cells remains a stem cell while the other starts to differentiate (Ulloa-Montoya et al., 2005) (Fig. 1.).

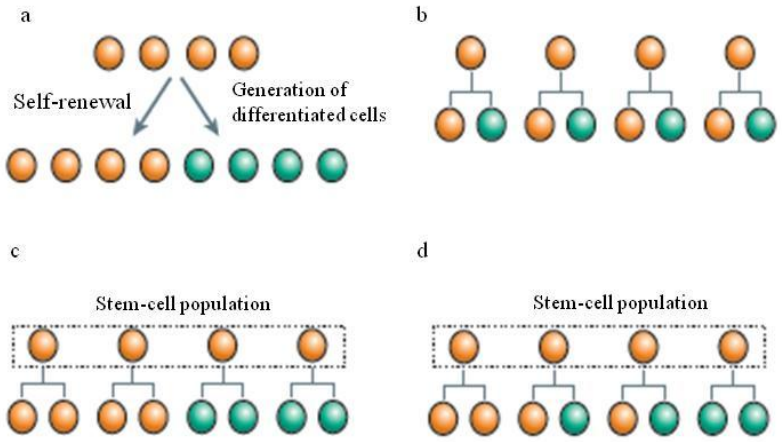

Figure 1. **Division strategies of stem cells** (Morrison & Kimble, 2006) **a**. Stem cells (orange) are able to self-renew and give rise to differentiated cells (green). **b-d.** Different strategies of stem cells to maintain a balance of stem cells and differentiated daughter cells. **b.** Asymmetric cell division: each stem cell generates one undifferentiated daughter stem cell and one daughter cell which starts to differentiate. **c.** Symmetric cell division: each stem cell can divide symmetrically to generate either two daughter cells or two differentiated cells. **d.** Combination of cell divisions: each stem cell can divide either symmetrically or asymmetrically.

In general, stem cells can be classified as either embryonic (ESC) or adult stem cells (ASC), depending on the developmental stage from which they were obtained (Fortier, 2005). The zygote up to the 8-cell stage of the morula is capable of forming the germ cells and cells of the

endo-, meso- and ectoderm layer, as well as the supporting trophoblast which is required for the survival of the developing embryo (Lakshmipathy & Verfaillie, 2005). These cells are therefore termed totipotent (Lakshmipathy & Verfaillie, 2005). Pluripotent stem cells are isolated from the inner cell mass of the blastocyst and give rise to endo-, meso- and ectoderm but not to extra-embryonic tissues. When unspecified, the term 'stem cell' typically refers to ESC (Fortier, 2005). Cells obtained from adult tissues which are capable of self-renewal and restricted differentiation into multiple organ specific cell types are termed multipotent ASC (Lakshmipathy & Verfaillie, 2005). A schematic overview of the classification of stem cells is given in Figure 2.

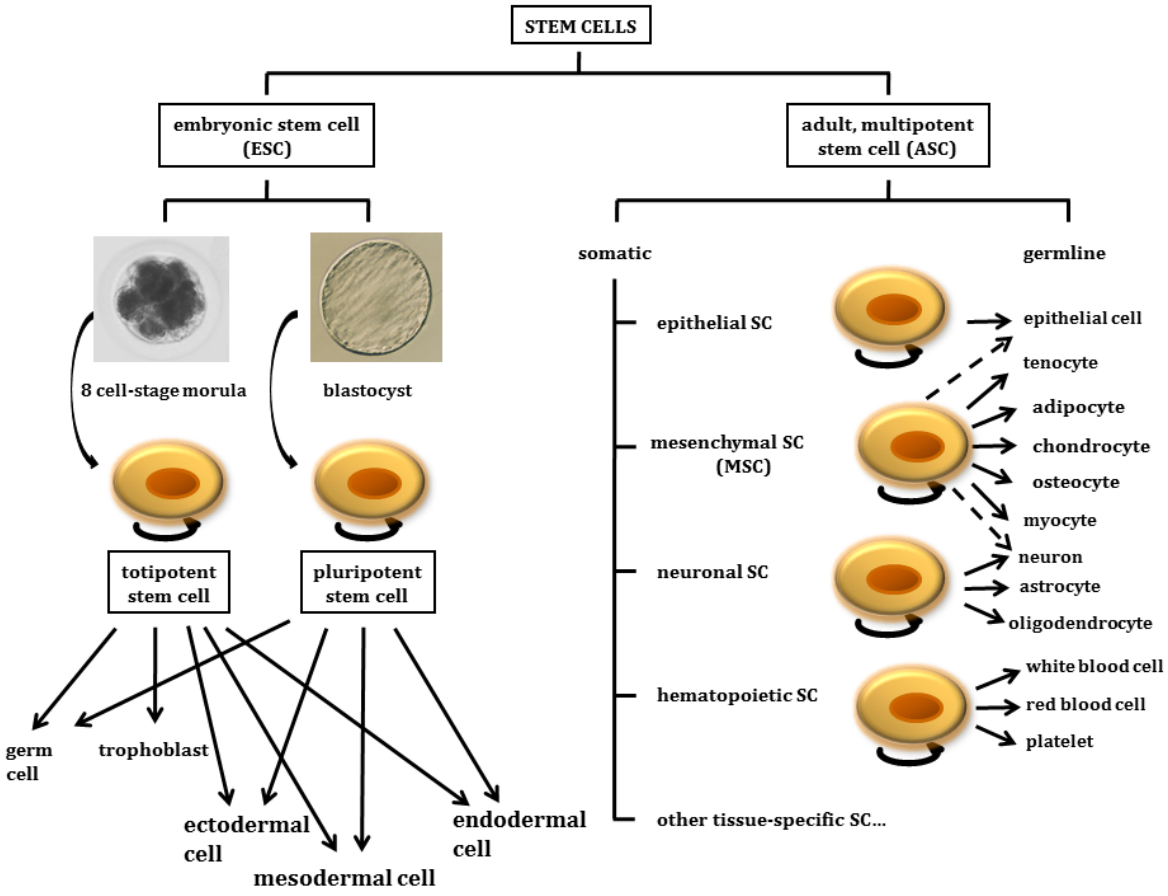

Figure 2. Classification of embryonic and adult stem cells.

Adult stem cells have traditionally been viewed as a resident population of cells within each tissue which are necessary to maintain organ mass during normal cellular turnover (Fortier, 2005). Examples include the hematopoietic stem cells that differentiate to all hematopoietic cells, the neural stem cells that give rise to neurons, astrocytes, and oligodendrocytes and the mesenchymal stem cells (MSC) that differentiate into fibroblasts, osteoblasts, chondroblasts, adipocytes, and skeletal muscle (Verfaillie et al., 2002). Interestingly, recent studies suggest that ASC have also differentiation potential into cell types of tissue lineages different from the tissue of origin, giving rise to the concept of stem cell plasticity (Fortier, 2005; Koch et al., 2008; Baer & Geiger, 2012). For example, hematopoietic stem cells have been reported to give rise to liver cells, and neural stem cells to early hematopoietic precursors (Lakshmipathy & Verfaillie, 2005).

1.2. Definition of mesenchymal stem cells

There are some ambiguities concerning the acronym "MSC". In the early 1990s, MSC meaning 'mesenchymal stem cell' was popularized although convincing data to support the stemness of these cells were lacking at that time. Therefore, the International Society of Cellular Therapy (ISCT) proposed that the term 'mesenchymal stem cell' should be reserved for those cells that (i) show long-term survival *in vivo*, (ii) have self-renewal capacities and (iii) possess the ability for tissue repopulation with multi-lineage differentiation *in vivo* (Horwitz et al., 2005). In contrast, plastic-adherent cells for which tri-lineage differentiation capacity *in vitro* can be demonstrated should be termed 'mesenchymal stromal cells'. However, the acronym MSC was kept for the latter cell population to minimize confusion in the field. As such, the biological properties of this cell population were indicated while the term 'stem' was eliminated from the nomenclature.

According to the ISCT, human MSC are characterized by three minimal criteria: (i) plastic-adherence when maintained in standard culture conditions, (ii) expression of a specific surface antigen panel, and (iii) multipotent differentiation potential (Dominici et al., 2006). Since MSC share many common features with endothelial, epithelial and muscle cells, a panel of antigens is necessary to unequivocally identify MSC (De Schauwer et al., 2011). Therefore, human MSC must express cluster of differentiation (CD)73, CD90, and CD105 and lack expression of CD14 or CD11b, CD34, CD45, CD79α or CD19, and major histocompatibility complex (MHC)-II (Dominici et al., 2006). Additional surface proteins which have been reported to be expressed by human MSC are Stro-1, CD13, CD29, CD44, CD106, and CD166 (Pittenger et al., 1999; Barry & Murphy, 2004; Kolf et al., 2007). However, the biological property that unequivocally characterizes MSC is their capacity to differentiate towards the osteogenic, chondrogenic, and adipogenic lineage, the so-called tri-lineage differentiation, using standard *in vitro* tissue culture-differentiating conditions (Dominici et al., 2006; Kolf et al., 2007).

1.3. Therapeutic potential of MSC in cell-based therapies

1.3.1. What is tissue engineering?

Tissue engineering has emerged as an interdisciplinary field in biomedical engineering with the goal of restoring the physiological structural architecture and biomechanical function of an injured tissue (Theoret, 2009; Borjesson & Peroni, 2011). Traditionally, in the so-called 'top-down' tissue engineering approach, a biodegradable polymeric scaffold serves as an adhesive substrate for seeded cells and supports the formation of the appropriate extracellular matrix and microarchitecture (Vertenten et al., 2009). However, it is often difficult to recreate the complex structural features of a tissue when using scaffolds (Nichol and Khademhosseini, 2009). In the

'bottom-up' approach on the other hand, biomimetic structures are constructed by designing structural features on a microscale, as such creating modular tissues which can be used as building blocks to construct larger tissues (Nichol and Khademhosseini, 2009). As cells are the expert builders of tissues and biomaterials, mechanisms by which cells engineer constructs and are able to manufacture the extracellular matrix, must be elucidated (Knothe Tate, 2011). For example, it has been demonstrated recently that MSC are extremely sensitive to mechanical signals, which are indispensable to build the microarchitecture of the engineered tissue, suggesting that MSC might be preferred cells to be applied in bottom-up approaches of for instance bone tissue engineering (McBride and Knothe tate, 2008).

An ultimate example of tissue engineering is a total joint replacement with a cell-based *in vitro* engineered joint that completely integrates and functions lifelong *in vivo* (Koch et al., 2009). Although this is still far from reality, it will be possible in the near future to obtain an improved repair, or maybe even regeneration, of focal defects such as traumatic cartilage injuries (Koch et al., 2009).

1.3.2. How do MSC function to heal tissues?

The significant therapeutic potential of MSC arises from their ability to promote tissue regeneration, prevent pathological scar formation, modulate immune responses and regulate inflammation (Borjesson & Peroni, 2011).

a. Primary tissue regeneration

Initially, the use of MSC for primary tissue regeneration was advocated based on their ability to differentiate into various tissue types (Stewart & Stewart, 2011). As such, the regeneration of

damaged tissues would be directly stimulated since injected MSC colonize the injury site, differentiate into the appropriate mesenchymal tissue type and affect repair (Stewart & Stewart, 2011). For example, it has been demonstrated that MSC, when applied within rat spinal cord injury models, differentiate into various neuronal cell types such as oligodendrocytes and astrocytes, and participate directly in the regeneration of the cord, as such reducing astrocytic scarring and improving functional outcome (Hill et al., 2004). Also in the repair of the cardiac muscle after infarction, MSC differentiate either into cardiomyocytes, as demonstrated in a mouse model (Fukuda, 2002), or into endothelial cells to support revascularization (Oswald et al., 2004). Moreover, they are able to fuse with the existing muscle cells to extend the longevity of the intrinsic cell population. Indeed, using dye coupling experiments, it was demonstrated that MSC and rat cardiomyocytes exhibited gap junctional communication as 18% of the single labeled MSC in co-culture rapidly converted into two-color positive cell formations (Rastan et al., 2004; Garbade et al., 2005). This process of cell fusion was futher confirmed by analyzing the chromosomal content of cultured stem cells and showing the co-expression of both donor as well as host specific cell markers in the same cell (Garbade et al., 2005).

However, some controversies still exist on the issue whether MSC primarily contribute to lesion healing by integrating into the injured tissue or indirectly by secreting immunomodulatory and bioactive trophic factors (Koch et al., 2008; Fortier & Travis, 2011). Firstly, the *in vitro* differentiation capacities of MSC towards different lineages were confirmed by assessing some qualitative aspects of tissue formation using histological stainings. However, it is obvious that the *in vivo* tissue-level complexities of e.g. mineralized bone or articular cartilage are far from realized *in vitro* (Stewart & Stewart, 2011). Thus, evidence of *in vitro* MSC differentiation does not guarantee their *in vivo* clinical usefulness. Along this line, it is noticed that the synthesis and

deposition of matrix proteins by MSC are generally far less distinct in comparison to the activities of differentiated chondrocytes. Nevertheless, the MSC chondrocyte-specific mRNA expression is often similar or even higher than that of intrinsic articular chondrocytes (Stewart & Stewart, 2011). Furthermore, there is evidence that MSC do not remain at the injury site after injection (Stewart & Stewart, 2011). In a study of Quintavalla et al. (2002), fluorescently-labeled MSC on a gelatin scaffold were implanted in full-thickness cartilage defects in goats and 14 days later, an extensive loss of the implanted MSC throughout the defect was observed. Fluorescent MSC were detected in the deeper regions of the defect as well as in the subchondral bone spaces, suggesting a migration of cells. On the other hand, it has been reported that MSC are guided by chemokines to migrate from their niche and home to sites of damaged tissue, although the exact nature of their signaling factors remains unknown (Kode et al., 2009). Guest et al. (2008a) demonstrated that fluorescent-labeled mesenchymal progenitor cells, which were injected into the superficial digital flexor tendon, mainly remained localized within the lesions although some labeled cells were present in healthy tendon surrounding the lesions, again indicating that migration does occur.

b. Anti-inflammatory and immunomodulatory activities of MSC

Generally, MSC are considered less immunogenic since they express low levels of MHC class I antigens and do not express MHC class II and co-stimulatory molecules such as CD40, CD80, and CD86 (Sensebe et al., 2009). These molecules are involved in the activation of T cells hence, cells that express MHC molecules can stimulate T cells directly if they display these costimulatory molecules (Klyushnenkova et al., 2005).

Activated MSC, primed by interferon-γ (IFN- γ), tumor necrosis factor-α, or other proinflammatory cytokines, can act both as anti-inflammatory and immunosuppressive agents in

response to injury (Tolar et al., 2010; Deuse et al., 2011; Peroni & Borjesson, 2011). The anti-inflammatory action of MSC is achieved by the production of bioactive mediators and adhesion molecules which reduce scar tissue formation and cell apoptosis, increase angiogenesis and stimulate the intrinsic cell population to regenerate function (Peroni & Borjesson, 2011). Immune responses are regulated by promoting shifts of type 1 T helper cells towards suppressive type 2 T helper cells and by inhibiting (i) antibody production by B cells, (ii) IFN-γ production from natural killer cells, (iii) dendritic cell maturation, and (iv) *in vitro* proliferation of T cells (Kode et al., 2009; Bunnell et al., 2010; Hoogduijn et al., 2010). The immunomodulatory effect of MSC is mediated either through direct cell-cell contact with cells of the immune system, or indirectly by the production of multiple soluble factors (Kode et al., 2009; Peroni & Borjesson, 2011). Direct cell contact between MSC and peripheral blood mononuclear cells (MNC) was demonstrated in a study of Quaedackers et al. (2009) where cell membrane interactions between MSC and all lymphocyte subsets were observed within one hour of co-culture, inhibiting the cytotoxic T cells and depleting the T helper cells from the cell suspension compartment. Soluble factors produced by MSC after stimulation, include: prostaglandin E_2, hepatic growth factor, transforming growth factor β, IFN-γ, interleukin-10, leukemia inhibitory factor, human leukocyte antigen G, and indoleamine 2,3-dioxygenase (Yagi et al., 2010; Deuse et al., 2011; Peroni & Borjesson, 2011). The immunomodulatory activities of MSC are schematically depicted in Figure 3.

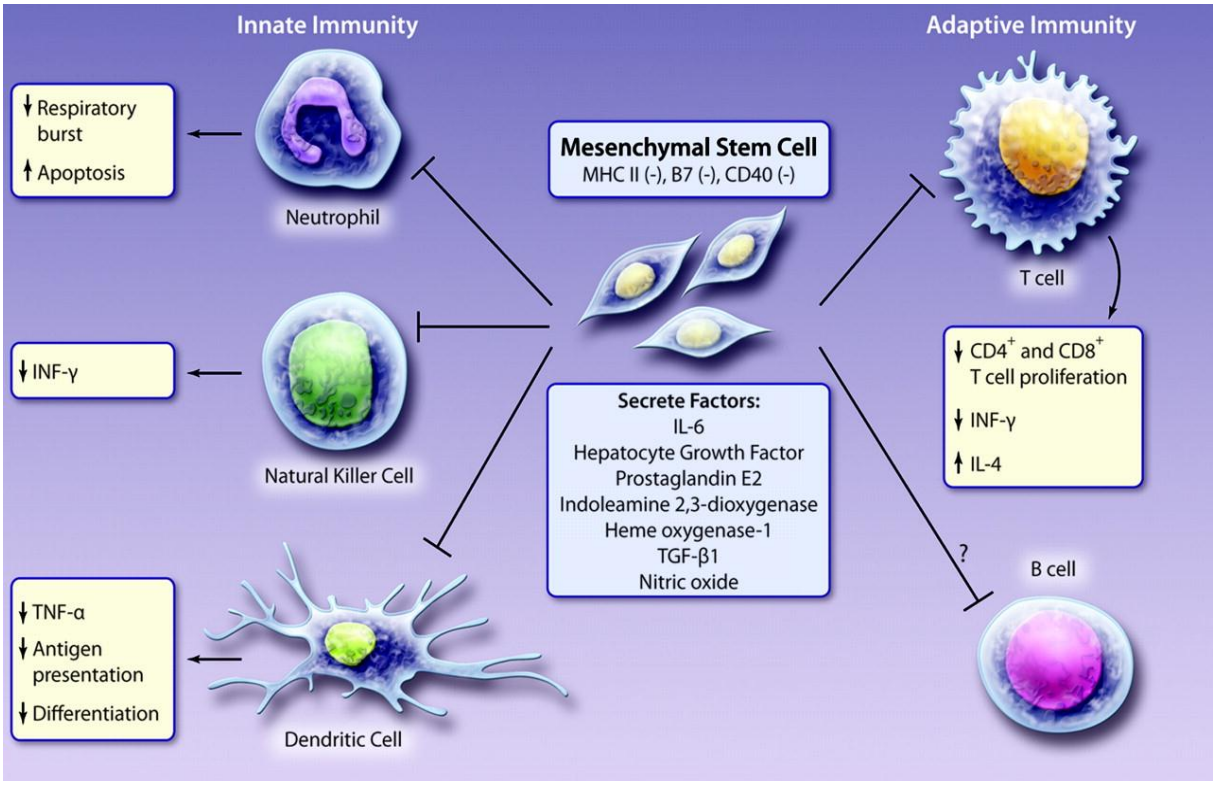

Figure 3. Immunomodulatory activities of MSC (Williams & Hare, 2011): a schematic overview.

2. Equine mesenchymal stromal cells

2.1. Characterization of equine MSC

2.1.1. Background

Unfortunately, and in contrast to human MSC, no uniform characterization criteria are available to date for MSC from animal origin in general, and equine origin in specific (Jiang et al., 2002; Dominici et al., 2006). As a result, the isolation and characterization of equine MSC has been proclaimed from several tissues, even though their isolation and identification was performed using different methods and/or a complete characterization was lacking (Table 1). While MSC from multiple species can easily be identified by their ability of plastic-adherence and tri-lineage differentiation, their surface antigen expression is not universally well-characterized (Dominici et al., 2006). A limited availability of species-specific or cross-reacting monoclonal antibodies (mAbs) in veterinary medicine hampers the possibilities for the proper immunophenotyping of MSC (Rozemuller et al., 2010). Indeed, a limited cross-reactivity of mAbs between species has recently been demonstrated by an extensive study of Ibrahim et al. (2007). In this study, over 379 mAbs against various human CD molecules were analyzed for cross-reactivity with equine leukocytes and only 14 of the mAbs tested (i.e. <4%) showed cross-reactivity in a cell-type-specific manner (Ibrahim et al., 2007).

Table 1. Overview of the different sources to obtain undifferentiated equine mesenchymal stromal cells and the characterization protocols used

Source	Authors	Cell surface markers	Gene markers	Differentiation potential
Bone marrow	Hegewald et al., 2004	ND	ND	C
	Koerner et al., 2006	ND	ND	O, C, A
	Vidal et al., 2006	ND	ND	O, C, A
	Arnhold et al., 2007	CD90	ND	O, C, A
	Cremonesi et al., 2008	ND	CD34	ND
	Giovannini et al., 2008	ND	ND	O, C, A
	Guest et al., 2008b	CD14, CD29, CD44 CD79α, CD90, MHC I, MHC II	ND	O, C, A
	Colleoni et al., 2009	ND	ND	O, C
	Violini et al., 2009	ND	CD34	O, T
	Radcliffe et al., 2010	CD11a/CD18, CD29, CD44, CD45RB, CD90	CD11a, CD13, CD29, CD44, CD45, CD90	O, C, A
Adipose tissue	Vidal et al., 2007	ND	ND	O, C, A
	Colleoni et al., 2009	ND	ND	O, C
	De Mattos Carvalho et al., 2009	CD13, CD44, CD90	ND	ND
Umbilical cord matrix	Hoynowski et al., 2007	CD34, CD45, CD54, CD73, CD90, CD105, CD133, CD146, MHC I	ND	O, C, A, N
	Cremonesi et al., 2008	ND	CD34	ND
	Passeri et al., 2009	ND	ND	O, C, A
Umbilical cord blood	Koch et al., 2007	ND	ND	O, C, A
	Reed and Johnson, 2007	ND	ND	O, C, A, M
	Shuh et al., 2009	CD18	ND	O, C, A
Peripheral blood	Koerner et al., 2006	ND	ND	O, C, A
	Giovannini et al., 2008	ND	ND	O, C, A
	Martinello et al., 2009	CD13, CD34, CD44, CD45, CD90, CD117, CD140a	ND	O, A, M

ND: not done; O: Osteogenic differentiation; C: Chondrogenic differentiation; A: Adipogenic differentiation; T: Tenogenic differentiation; N: Neuronal differentiation; M: Myogenic differentiation

The state of the art on the characterization of equine MSC obtained from different sources will be briefly discussed in the following subchapters. Hereby, the general purpose of refining the mesenchymal nomenclature is to establish well-determined definitions in equine veterinary medicine, allowing an accurate comparison of research data obtained by multiple investigators (Koch et al., 2009).

2.1.2. Characterization of undifferentiated equine MSC

a. Morphological characterization

Friedenstein et al. (1976) were the first to isolate murine MSC in the seventies using their ability to adhere to tissue culture plastic. Plastic-adherence is a common characteristic for all isolated potential MSC populations (Taylor et al., 2007). Hereby, putative MSC, including equine MSC, are morphologically identified as spindle-shaped cells that grow in a monolayer and show a varying cellular morphology, from very slender and elongated towards more cuboidal with shorter cytoplasmatic extensions (Koch et al., 2007 and Fig.4).

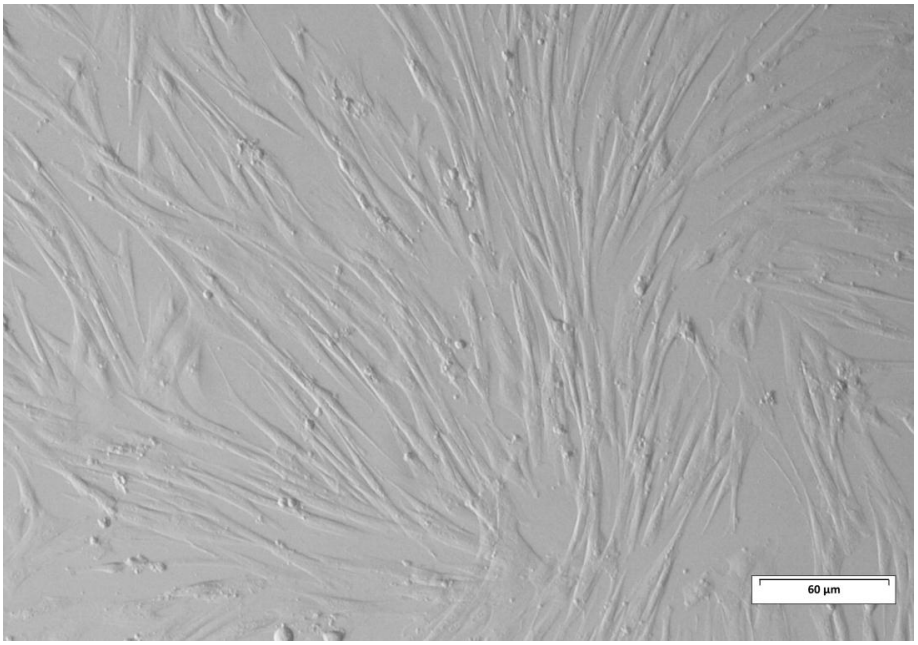

Figure 4. Equine MSC. A light microscopic image of a monolayer of undifferentiated, plastic-adherent and spindle-shaped equine MSC in culture (40x).

b. Gene expression characterization

Characterization of MSC at the mRNA level is a valuable alternative when no (cross-reacting) mAbs are available to characterize MSC at the protein level. However, as an altered gene expression not always translates into an actual difference at the post-translational protein level (Greenbaum et al., 2003), such analyses should be interpreted with care. Still, the group of Radcliffe et al. (2010) has recently studied the temporal expression changes of several genes during establishment of equine MSC cultures, both at the mRNA as well as at the protein level. Hereby, it was found that at all culture time points tested, the gene expression followed the same pattern as the cellular protein expression, indicating that mRNA analysis can still be of great value, for instance when suitable mAbs are lacking (Radcliffe et al., 2010).

In human stem cell research, gene expression is frequently used to characterize undifferentiated MSC and the most commonly studied genes are pluripotency markers which are normally used to characterize ESC (Ginis et al., 2004). Hereby, it was found that human MSC express Nanog, Oct4 and Sox2 (Ginis et al., 2004; Boyer et al., 2005). These pluripotency markers have also been analyzed by qRT-PCR for equine MSC and in line with human MSC, equine MSC are positive for these 3 markers (Li et al., 2006; Cremonesi et al., 2008; Violini et al., 2009).

The pluripotency markers Nanog, Oct4 and Sox2 are transcription factors which are defined as regulatory proteins binding specific short DNA sequences upon activation, thereby controlling gene transcription either positively or negatively (Latchman, 1997). These transcription factors form the core regulatory network that ensures (i) the suppression of genes that lead to differentiation of ESC and (ii) the maintenance of pluripotency of these cells (Guest et al., 2007; Reed & Johnson, 2008). Oct4, also designated as POU5F1, is known to be essential for the

formation of pluripotent stem cells in the early embryo since it is required for self-renewal and multi-lineage differentiation (Guest et al., 2007; Violini et al., 2009). Sox2 and Nanog on the other hand are transcription factors that govern pluripotency (Reed & Johnson, 2008).

c. Immunophenotypical characterization

In general, immunophenotyping of human MSC is mostly performed by flow cytometry, as this technique allows for a rapid identification of cells. Hereby, it is mandatory that a panel of cellular protein markers is analyzed to assure that the selection of a putative MSC population is not confounded by the presence of other cells, e.g. hematopoietic stem cells. Ideally, a multicolor flow cytometric assay should be used to identify the individual cells co-expressing different MSC markers and lacking the expression of haematopoietic antigens (Dominici et al., 2006). However, no such multicolor flow cytometry approach has been reported for equine MSC characterization.

Several pluripotency markers which are used to immunophenotype human ESC, are the cell surface glycolipids SSEA-1, SSEA-3 and SSEA-4, and the extracellular matrix keratan sulfate antigens Tra-1-60 and Tra-1-81, which are all surface antigens present in certain subpopulations of human MSC too (McGuckin et al., 2005; Zhao et al., 2006; Gang et al., 2007; Sun et al., 2007; Xu et al., 2009). Recently, the expression of these proteins has been tested in undifferentiated equine MSC also using immunocytochemistry and flow cytometry. However, the results obtained by using mAbs against these pluripotency markers are conflicting (Table 2). Indeed, for SSEA-1, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81, protein expression in equine MSC has been reported (Hoynowski et al., 2007; Reed & Johnson, 2008) as well as denied (Guest et al., 2008b) (Table 2). For the other pluripotency marker, Oct4, two research groups have demonstrated its

Table 2. Expression of cellular protein markers on human versus equine undifferentiated MSC.

Cellular protein marker	Synonymes	Expression in		Positive control
		Human MSC	Equine MSC	
Nanog		+	?	Equine blastocysts
Oct4	POU5F1	+	+/-	Equine blastocysts
Sox2		+	?	Equine blastocysts
SSEA-1		-	+/-	Equine blastocysts
SSEA-3		+	+ /-	Equine blastocysts
SSEA-4		+	+ /-	Equine blastocysts
Tra-1-60		+	+ /-	Equine blastocysts
Tra-1-81		+	+ /-	Equine blastocysts
CD29	β1-integrin	+	+	T &B cells, Mo
CD44		+	+	Mo, granulocytes, lymphocytes
CD73	Ecto 5'nucleotidase, SH3, SH4	+	?	T and B cells
CD90	Thy-1	+	+	T cells, thymocytes, neurons, EC and fibroblasts
CD105	Endoglin, SH2	+	+	EC
CD106	VCAM-1	+	?	EC after stimulation by cytokines
CD166	SB-10, ALCAM	+	?	Thymic Ep C, activated T cells, B cells, Mo
MHC I	HLA-I	+	+	Leukocytes
CD11b	ITGAM, CR3A	-	?	Mo, Mϕ
CD14		-	-	Mo, Mϕ
CD19		-	?	B cells
CD34		-	-	Primitive HP and EC
CD45	LCA, B220, T200	-	-	All leukocytes
CD79α		-	-	B cells
MHC II	HLA-DR, HLA-II	-	-	Lymphocytes

+ present; - absent; ?: unknown; Mo: Monocytes; Mϕ: Macrophages; EC: Endothelial cells; Ep C: Epithelial cells; HP: Hematopoietic progenitors. Cells from the positive control group can be used to validate cross-reactivity when no anti-horse Abs are available.

expression on equine MSC at the protein level (Hoynowski et al., 2007; Reed & Johnson, 2008), while the group of Guest et al. (2008b) was unable to observe Oct4 expression (Table 2).

Aside from these pluripotency markers, the other cell surface and intracellular markers used for immunophenotyping MSC typically belong to the 'CD' group (Table 2). The CD nomenclature is based on a protocol used for the identification of cell surface glycoprotein molecules present on leukocytes. The Human Leukocyte Differentiation Antigens workshops were initiated to define and compare the specificities of the mAbs by determining their expression on specific cell lineages (Lunn, 1993). Antibodies were coded and sent to different participating laboratories for blind analysis. After collection, data were analyzed using the statistical procedure of 'cluster analysis' which identified clusters of antibodies with very similar binding patterns to leukocytes at various stages of differentiation (Zola et al., 2005). Hence, the 'cluster of differentiation' or CD nomenclature was born. Since 2004, the focus has been extended (i) from leukocytes to other cell types such as endothelial and stromal cells and (ii) from cell-surface molecules to any molecule, meaning that molecules with an intracellular localization are also recognized as markers of differentiation (Zola et al., 2005).

According to the recommendations of the ISCT, human MSC should be positive for certain markers and negative for others (Table 2). Important cellular protein markers for which MSC should be positive include CD29, CD44, CD73, CD90, CD105, CD106, CD166 and MHC I, whereas at the same time they should be negative for CD11b, CD14, CD19, CD34, CD45, CD79α and MHC II (Pittenger et al., 1999; Barry & Murphy, 2004; Horwitz et al., 2005; Dominici et al., 2006). In general, the majority of CD molecules functions as (i) receptors or ligands or (ii) plays a role in the cell signaling pathways, antigen recognition or antigen presentation.

Positive markers include members of the integrin family such as the β1 integrins (CD29), also known as the Very Late Activation antigens, which are mainly involved in cell adhesion mechanisms (Lunn, 1993). The hyaluronate receptor CD44 is an example of a non-integrin, which is important for the adhesion of various leukocytes to endothelia and plays a role in T-lymphocyte activation (Lunn, 1993; Mitchell et al., 2006). Although both CD29 as CD44 are some of the most consistently expressed MSC markers across species, these proteins are expressed by multiple cell types in many tissues, and therefore, their usefulness as MSC marker might be limited (Boxall & Jones, 2012). The CD73 protein, also known as ecto-5'-nucleotidase, is a purine catabolic enzyme with broad substrate specificity (Resta & Thompson, 1997). It is known to be involved in BM stromal interactions, MSC migration as well as in the MSC adaptive immunity modulation (Boxall & Jones, 2012). Activation of CD90 promotes T cell activation and regulates different biological processes like cell-cell and cell-matrix cellular interactions in axon regeneration, apoptosis, adhesion, migration, cancer and fibrosis (Rege & Hagood, 2006). The CD105 molecule, also called endoglin, is a type I membrane glycoprotein which is a part of the transforming growth factor-β complex and plays a role during MSC chondrogenic differentiation (Sanz-Rodriguez et al., 2004). During culture, MSC are reported to be uniformly positive for the 3 latter markers, i.e. CD73, CD90 and CD105, at similar levels in early-passage as in late-passage MSC (Boxall & Jones, 2012). However, both CD73 and CD105 are also expressed on skin fibroblasts and umbilical vein endothelial cells which implies that the demonstration of CD73 and CD105 without CD90 is insufficient to prove their MSC identity (Boxall & Jones, 2012). The cell adhesion molecule CD106 mediates the adhesion of leukocytes following activation by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (Barreiro et al., 2002). The expression of this protein declines at later passages and is

also downregulated after MSC differentiation, suggesting that this marker might be indicative for the most undifferentiated cells within expanded MSC cultures (Boxall & Jones, 2012). The CD166 protein is also known as the activated lymphocyte common adhesion molecule (Mitchell et al., 2006). The MHC antigens (MHC I and MHC II) are especially known from their role in graft rejection. They present the antigen to T-lymphocytes, which recognize processed antigens only when they are associated with MHC I or MHC II molecules (Lunn, 1993).

Negative markers include the CD11b molecule which is an adhesion protein important for the myeloid cell extravasation and serves also as complement receptor (Lunn, 1993). The CD14 protein induces the synthesis of tumor necrosis factor by monocytes (Lunn, 1993). The earliest cell surface molecule related to the B cell lineage differentiation, is CD19. It provides a costimulatory signal for activation through the B cell receptor (Horvath et al., 1998). The CD34 protein is a sialomucin and L-selectin ligand, which is associated with hematopoietic stem cells (Mitchell et al., 2006). Nevertheless, a small fraction of human bone marrow (BM)-derived MSC has been reported to be positive for CD34 (Simmons & Torok-Storb, 1991a; Zvaifler et al., 2000). These studies, however, were performed using BM-derived MSC before culture. It appeared that these cells lost their CD34 expression upon *in vitro* culture, which is identified as a consistent feature of stromal cells (Simmons & Torok-Storb, 1991b; Zvaifler et al., 2000; Copland et al., 2008). Furthermore, it was recently demonstrated that also 13.7% of the human plastic-adherent cells isolated from adipose tissue (AT) were CD34 positive and simultaneously CD45 negative (Quirici et al., 2010). Moreover, these isolated cells were unable to give rise to hematopoietic colonies under specific *in vitro* culture conditions, indicating that the hematopoietic origin of these CD34 positive cells could be excluded (Quirici et al., 2010). Similar with the studies reported for human BM-derived MSC, the CD34 expression of MSC

from AT was also progressively down-modulated during culture (Quirici et al., 2010). The CD45 molecule, also called pan-leukocyte marker, belongs to the protein tyrosine phosphatase family, which is a group of signaling molecules regulating cellular processes like cell growth, differentiation, mitosis and oncogenic transformation. The expression of CD45 is necessary for signal transduction via the B cell antigen-receptor complex (Brown et al., 1994).

For human MSC, strict guidelines are proposed by the ISCT concerning the presence or absence of these markers as briefly mentioned sub 1.2. Unfortunately, no such guidelines are available for equine MSC to date. Nevertheless, we strongly support an unequivocal immunophenotypical characterization of equine MSC as this would largely reduce the discrepancies reported by different research groups. The lack of proper control groups and the use of different commercial mAbs can be possible explanations for conflicting results since it has been reported that the protein expression patterns of some pluripotency markers in ASC can be antibody-dependent, i.e. different commercial clones of antibodies against the same antigen can result in different outcomes in expression (Zuk, 2009).

2.1.3. Characterization of differentiated equine MSC

Differentiation is a process which explicitly changes the cell's size, shape, membrane potential and metabolic activity caused by modifications in gene expression. The capacity of MSC to differentiate towards the osteogenic, chondrogenic and adipogenic lineage is one of the hallmarks of MSC (Dominici et al., 2006). An overview of the different methods to characterize differentiated equine MSC is given in Table 3.

a. Induction of differentiation

Under specific culture conditions, MSC have a multi-lineage differentiation potential with mainly osteogenic, chondrogenic and adipogenic differentiation capacities (Table 3). Using standard induction media, equine MSC derived from bone marrow (BM), adipose tissue (AT), umbilical cord matrix (UCM), umbilical cord blood (UCB) or peripheral blood (PB) can be induced to differentiate towards both osteogenesis and chondrogenesis (Vidal et al., 2006; Hoynowski et al., 2007; Koch et al., 2007; Giovannini et al., 2008; Guest et al., 2008b). Their adipogenic differentiation, however, appeared less straightforward and the standard assays developed for human MSC had to be optimized for equine MSC. More specifically, rabbit serum was found to be necessary to induce adipogenesis in equine MSC (Vidal et al., 2006; Koch et al., 2007; Giovannini et al., 2008).

Table 3. Overview of the methods used to characterize differentiated equine MSC

Differentiation lineage	Histological staining	Gene expression	Protein expression
Osteogenic	Alkaline phosphatase activity Alizarine Red S Von Kossa	Runx2 Osteonectin SPP1	Runx2 Collagen I Osteocalcin β_1 integrin Osteonectin
Chondrogenic	Toluidine Blue Alcian Blue Saffranin O Masson trichrom	Sox-9 Collagen II Collagen II/collagen I Aggrecan/Versican	Collagen II β_1 integrin
Adipogenic	Oil Red O	PPAR-γ	Adiponectin β_1 integrin
Myogenic	HE Masson	Desmin	Desmin Phalloidin Myf5 MyoD Smooth muscle Actin
Tenogenic	HE	Tenomodulin Decorin	ND

HE: Hematoxylin and eosin staining; ND: not done

In addition, myogenic, tenogenic and even neuronal and hepatogenic differentiation potential has been reported for equine MSC. Reed and Johnson (2008) and Martinello et al. (2009) succeeded in differentiating equine MSC isolated from the UCB and PB, respectively, towards muscle cells, whereas the former group also performed differentiation towards the hepatogenic lineage. Equine MSC isolated from the BM were also able to differentiate towards tenocytes (Violini et al., 2009), while Hoynowski et al. (2007) could differentiate MSC isolated from the UCM towards the neuronal cell lineage. The latter shows the capability of equine MSC to transdifferentiate into multiple germ layers, since neuronal cells belong to the ectoderm in contrast to the other lineages which belong to the mesoderm, and are hence a good example of the plasticity of ASC.

b. Morphological and histological characterization

Increased alkaline phosphatase activity and matrix stainings to identify phosphate and calcium deposits in tissue sections, such as Von Kossa and Alizarine Red S, respectively (Fig. 5A&B), are often used to demonstrate osteogenesis (Dominici et al., 2006). Safranin O, Alcian blue and Toluidine blue stainings confirm chondrogenesis by staining acidic mucosubstances and acetic mucins (Dominici et al., 2006) (Fig. 5C). Masson's Trichrome staining can demonstrate the collagen synthesis when chondrogenic differentiation is performed (Giovannini et al., 2008). The Oil Red O staining is used to identify adipogenesis since it stains neutral triglyceride droplets (Dominici et al., 2006; Arnhold et al., 2007) (Fig. 5D).

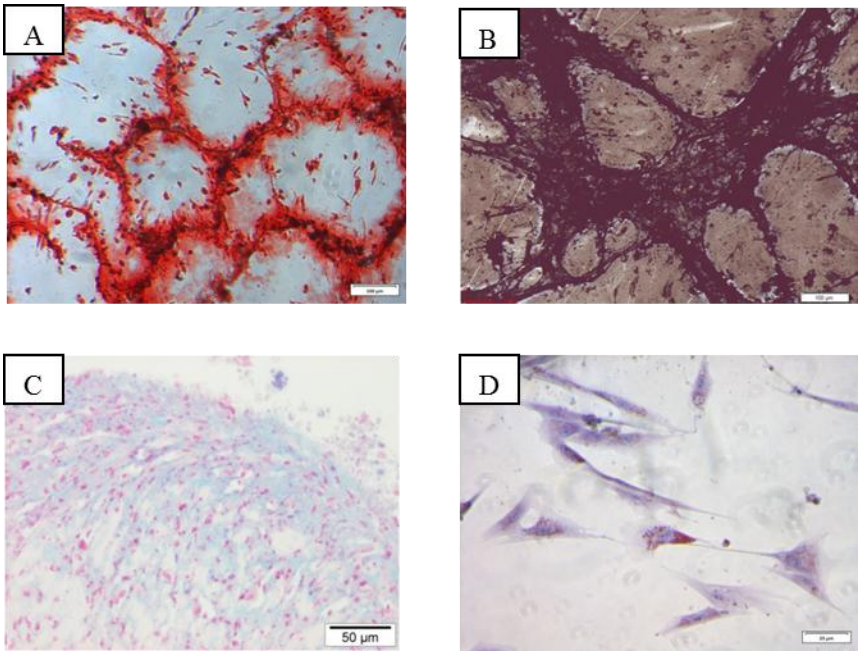

Figure 5. Differentiated equine MSC isolated from umbilical cord blood. A light microscopic image of MSC differentiated towards the osteogenic lineage as confirmed by Alizarine Red S (A,10x) and Von Kossa staining (B,10x), towards chondrogenic lineage as confirmed by Alcian Blue staining (C,40x) and towards adipogenic lineage as confirmed by Oil Red O staining (D,40x).

c. Gene expression and immunophenotypical characterization

Besides histology, a complementary approach to evaluate the differentiation capacity of MSC is to perform RT-PCR for gene expression analysis of specific genes or transcription factors characteristic for each particular cell lineage. For equine MSC, osteogenic differentiation is generally confirmed by the detection of Runx2, also known as Cbfa-1, which is a specific transcription factor for early osteogenesis, and of osteonectin, a calcium-binding glycoprotein that interacts with hydroxyapatite and initiates the mineralization of cartilage and bone (Guweidhi et al., 2005; Reed & Johnson, 2008). Collagen I is also correlated with early osteoblast expression since it forms the network on which mineralization starts, while osteocalcin is a non-collagenous protein specific for late osteoblast expression (Declercq et al.,

2005). To identify chondrogenic differentiation, gene expression levels of Sox9, collagen II and aggrecan are evaluated (Reed & Johnson, 2008). PPAR-γ expression is detected to confirm adipogenic differentiation since it is an adipogenic-specific transcription factor that stabilizes the metabolic function of differentiated adipocytes (Pittenger et al., 1999; Lee et al., 2004; Wang et al., 2004). Adiponectin is the most abundant protein in adipose tissue and its expression is also used to confirm adipogenic differentiation (Csaki et al., 2007).

For the immunophenotypical characterization of differentiated equine MSC, several cell surface markers have been described and proposed, mostly extrapolated from human medicine research (Table 3). However, surface antigen expression is not universally well characterized between species like e.g. human, mouse, horse or dog (Dominici et al., 2006).

2.2. Sources of equine MSC

To date, equine MSC have been isolated from different sources including, but not limited to bone marrow (BM) (Smith et al., 2003), adipose tissue (AT) (Braun et al., 2010), umbilical cord blood (UCB) (Koch et al., 2007), peripheral blood (PB) (Koerner et al., 2006) and fetal adnexa (Lange-Consiglio et al., 2011). So far, clinical studies have been reported using MSC derived from the former three sources and therefore, these will be discussed in detail.

a. Bone marrow

As MSC were originally isolated from BM, this source is the most studied and hence, the best characterized to date (Fortier & Travis, 2011). The sternum is commonly used to aspirate BM although alternative sites such as tuber coxae, tibia and humerus have been described as well (Taylor & Clegg, 2011). Harvesting BM is a highly invasive procedure which has potential drawbacks such as low cell yield, pain associated with the collection and even pericardial

laceration during collection (Nixon et al., 2008). Besides, safety concerns for both the patient and the clinician must be considered when harvesting BM from adult horses (Berg et al., 2009). Furthermore, three to six weeks of culture after the isolation of BM-derived MSC are required to allow cellular expansion, in order to obtain a sufficient number of MSC for treatment (Berg et al., 2009; Fortier & Travis, 2011).

b. Adipose tissue

Most commonly, AT can be collected from the tail head of horses, using a less invasive procedure compared to BM, or from the dorsal gluteal muscles, the inguinal and sternal fat depots (Nixon et al., 2008; Taylor & Clegg, 2011). In highly trained athletic horses, the collection can be difficult because of the small amount of accessible fat (Carrade et al., 2011a). Adipose tissue yields higher numbers of MSC per unit volume than BM (Toupadakis et al., 2010). Usually, a mixture of cells isolated from the AT are injected into the patient without a cell culture step, giving the advantage of supplying cells within 48 hours (Fortier & Travis, 2011).

c. Umbilical cord blood

Umbilical cord blood is a non-invasive source for MSC that easily can be collected at parturition before the umbilical cord ruptures (Carrade et al., 2011a; Taylor & Clegg, 2011). In earlier times, there was the wide-spread belief that the mare was still passing blood towards the foal through the umbilical cord immediately after parturition (Bartholomew et al., 2009). However, using Doppler ultrasound, no blood flow was detected through the cord which means that UCB can be collected without harming the foal (Doarn et al., 1987). As reported by Carrade et al. (2011a), high numbers of MSC can be expanded from UCB. Furthermore, these MSC are immediately available at the time of the injury although this implies their long-term frozen storage in liquid nitrogen (Berg et al., 2009).

d. Comparison of MSC characteristics derived from the principal sources

For human UCB-derived MSC, it has been demonstrated that they have the highest expansion potential, longest telomere length and a broader differentiation potency, when compared to BM-derived or AT-derived MSC (Kogler et al., 2004; Kern et al., 2006; Shuh et al., 2009). Likewise equine UCB-derived MSC have been reported to be highly proliferative, multipotent and to display a delayed senescence compared to BM-derived MSC (Carrade et al., 2011a). However, it has recently been shown that cell yield as well as proliferation were significantly higher for equine AT-derived MSC compared to BM- or UCB-derived MSC (Vidal et al., 2012; Burk et al., 2012). An age-related decline in the quantity of BM-derived MSC has been reported for several species, and also for AT-derived MSC, a correlation between age-related decrease in their quantity and impairment in self-renewal differentiation capacities was recently described (Alt et al., 2012). Furthermore, AT- and UCB-derived MSC migrate faster indicating that their graft integration *in vivo* might be better than that of BM-derived MSC (Burk et al., 2012). The migrating and homing abilities of MSC are essential features during the tissue regeneration process (Li et al., 2009).

Multi-lineage potential is evident for all MSC isolated from these three principal sources (Borjesson & Peroni, 2011), although significant differences have been recently reported for osteogenic and chondrogenic potential (Burk et al., 2012). The BM-derived MSC possess the highest *in vitro* osteogenic potential (Toupadakis et al., 2010; Burk et al., 2012) whereas chondrogenic differentiation is most prominent in UCB-derived MSC (Berg et al., 2009; Burk et al., 2012). Conflicting results are obtained for the chondrogenic differentiation potential of BM-derived MSC: a study of Burk et al. (2012) showed the weakest chondrogenic potential while a study of Giovannini et al. (2008) observed an intense chondrogenic differentiation, even superior

to that of AT-derived MSC (Vidal et al., 2008). Concerning the latter MSC, the osteogenic as well as the chondrogenic differentiation capacities are less distinct (Vidal et al., 2008; Toupadakis et al., 2010). There is some scientific evidence that the differentiation capacities of AT-derived MSC might vary with the anatomical location (Stewart, 2011). As such, MSC derived from intra-articular fat depots are reported to exhibit substantially more chondrogenic potential than MSC isolated from non-articular AT (Stewart, 2011). Despite the lower differentiation potential of AT-derived MSC, they are well known as particularly potent immunomodulatory agents and as such might be specifically indicated for anti-inflammatory and immunosuppressive applications (Stewart & Stewart, 2011). Finally, when compared to BM- or AT-derived MSC, UCB-derived MSC might be more committed to stimulate angiogenesis which is important in the bone-healing cascade (Toupadakis et al., 2010).

In conclusion, when applying stem cell therapy, the choice of the MSC source certainly plays a role and may vary depending on the type of injury for which a treatment is desired, since some healing properties of MSC are influenced by tissue source (Borjesson & Peroni, 2011).

2.3. Mesenchymal stromal cells in equine veterinary medicine

2.3.1. General considerations

The therapeutic use of equine MSC for orthopedic injuries has been described at first in 2003, with less than five peer-reviewed fundamental research articles published at that time (Borjesson & Peroni, 2011). Since then, the clinical use of MSC has been exploding with thousands of horses now being treated worldwide. Although the fundamental research has also expanded, it lags substantially behind when considering the rapid product development and clinical

experimentation using equine MSC (Borjesson & Peroni, 2011). It must be mentioned, however, that many of the disorders that seem ideally suited for MSC treatment, have a long history of potential revolutionary therapies which have subsequently been shown to be either not efficacious or even deleterious for the animals' recovery (Clegg & Pinchbeck, 2011).

The efficacy of equine MSC therapy is difficult to evaluate since the use of appropriate control groups is not always included and this treatment is often combined with other biological factors such as BM supernatant, autologous serum, platelet-rich plasma, etc. (Koch et al., 2009). Nevertheless, demonstrating true efficacy should be a collective goal of the equine veterinary community using clinical trials which include sufficient and similar cases and a consistent and standardized panel of objective outcome measures (Stewart, 2011). For example, magnetic resonance imaging, radiology and ultrasonography are possible tools to evaluate reparative responses in tendon, ligament, bone, and articular lesions, while force plate and gait analysis measurements can provide quantitative data of functional recovery to confirm the clinicians' diagnostic findings and return-to-competition results (Stewart, 2011). The current clinical literature frequently relies on study designs that often do not respond to the gold standard of evidence-based medicine, i.e. blinded randomized control trials. Indeed, the latter set-up is difficult to undertake in equine veterinary science because of the logistical and economical hurdles of such a study (Clegg & Pinchbeck, 2011). Although several studies are controlled, the experimental power is often lacking because of the limited sample size in horse-based studies and the inter-animal variability of the pathological conditions (Clegg & Pinchbeck, 2011).

Considering both short-term and in particular long-term safety of stem cell therapy, little information is available as no central regulatory body is overlooking the current empirical use of equine MSC (Koch et al., 2009). In some studies, it was verified whether or not possible adverse

reactions could be noticed. In 2003, Smith et al. performed the first reported MSC therapy implanting autologous culture-expanded BM-derived MSC into spontaneously occurring core lesions of the superficial digital flexor tendon. No adverse reactions like increased thickening of the injured region or disruption of the tendon were detected at 10 days nor at 6 weeks after implantation (Smith et al., 2003). Peritendinous mineralization after treatment with BM-derived MSC has been reported only once on more than 1500 treated clinical cases (Alves et al., 2011).

Most equine clinical studies report on the use of BM-derived MSC which have been expanded *in vitro* prior to *in vivo* use, hereby providing a certain degree of quality control and the expectation that the obtained effect of the treatment is actually caused by the MSC (Koch et al., 2009). On the other hand, cell suspensions containing a mixture of cells can also be administered immediately without an *in vitro* expansion step, which reduces the *in vitro* selection pressure on the cells and is less time-consuming (Koch et al., 2009). It is not known yet how many MSC are present in these non-expanded cell suspensions, nor if there is a critical number of MSC required to induce regeneration (Berg et al., 2009). So far, almost no dose-response studies have been performed (Fortier & Travis, 2011). In a study of Pacini et al. (2007), autologous BM-derived MSC were used to treat non full-thickness lesions of the superficial digital flexor tendon. One of the treated horses received less than 1×10^6 MSC and showed no healing of the tendon in contrast with the other treated horses which received on average 9.5×10^6 MSC and displayed an excellent ultrasound image of the tendons (Pacini et al., 2007). These preliminary findings seem to suggest that there might indeed be an optimal number of MSC for treatment.

In summary, it is apparent that many fundamental questions remain to be answered regarding the clinical use of MSC in equine medicine, such as the efficacy of treatment, the MSC dose, the tissue source, the route of administration, whether scaffolds are necessary or not, the timing of

administration, and the use of autologous or allogeneic MSC (Borjesson & Peroni, 2011; Fortier & Travis, 2011).

2.3.2. Autologous versus allogeneic MSC

At present, most cell-based therapies in horses are using autologous MSC. Nevertheless, an allogeneic source would provide an off-the-shelf, more standardized and readily available product without the inherent lag period associated with isolation and expansion of autologous MSC (Alves et al., 2011). Although no costs are associated with the harvest procedure, it is still questionable if the use of allogeneic MSC is more cost-effective since there are additional storage expenses and extra tests needed to ensure that no infectious diseases are transmitted (Fortier & Travis, 2011). Some preliminary studies suggest that allogeneic MSC can be used without eliciting an apparent cell-mediated immune response in horses. In a study of Guest et al. (2008a), three lesions in the superficial digital flexor tendon were created and 7 days later, either BM supernatant alone, or autologous or allogenic BM-derived MSC were administered. These two types of MSC were transfected with green fluorescent protein to allow their monitoring after injection (Guest et al., 2008a). Haematoxylin-eosin staining revealed no qualitative differences between the control and the MSC-injected lesions (Guest et al., 2008a). Staining with an anti-green fluorescent protein Ab confirmed that the majority of cells remained localized within the lesions, although some labeled cells were observed in the surrounding healthy tendon, suggesting a certain degree of migration. However, these cells displayed a typical tenocyte morphology and were well integrated into the tendon pattern. Furthermore, the injection of allogeneic BM-derived MSC did not cause any observable cell mediated immune response from the host, based on the absence of external symptoms of inflammation and the lack of histological differences in the

density of leukocytes between the autologous and allogenic implantation sites as criteria (Guest et al., 2008a).

To evaluate possible acute graft rejections or delayed-type hypersensitivity responses, equine allogeneic MSC, derived from UCM, were injected intradermally in six horses in the study of Carrade et al. (2011a). Four test conditions (autologous MSC, allogeneic MSC, positive and negative control) were compared evaluating the injection sites for the presence of erythema and measuring both their horizontal and vertical wheal diameters. A mild, self-resolving response to the injection of both autologous as allogeneic MSC was detected when compared to the negative control group, consisting of saline injections. In a second study from the same group, the effects of injecting autologous, related allogeneic or unrelated allogeneic UCB- or UCM-derived MSC in healthy equine joints, were determined (Carrade et al, 2011b). Although a single intra-articular injection of MSC elicited an inflammatory reaction in a non-injured joint, no significant difference was demonstrated either in the type or the degree of inflammation using autologous, related or unrelated allogeneic cells. These findings confirm that allogeneic MSC may be both safe and a practical alternative treatment option for equine orthopedic injuries.

Nevertheless, it should be emphasized that the available studies included only a limited number of horses and a very short-term monitoring period. Therefore, larger controlled clinical studies with a longer follow-up period to evaluate the outcome are necessary to ensure the safety and efficacy of equine allogeneic MSC use (Koch et al., 2009).

2.3.3. Clinical applications of equine MSC

At present, MSC are mainly used in veterinary medicine to treat musculoskeletal diseases in marked contrast to human medicine where MSC therapies are primarily focused on immune-mediated, inflammatory and ischemic diseases (Borjesson & Peroni, 2011). However, horses also suffer from aforementioned non-orthopedic diseases. Therefore, the use of MSC in equine veterinary medicine could be considerably expanded (Fig. 6).

Furthermore, a general consideration is that the horse is accepted as a suited animal model for several human cell-based therapies. In human medicine, preclinical efficacy and safety testing of for example new treatments to enhance bone repair, must be evaluated using 2 animal species, i.e. rats and a second non-rodent large animal that has a similar bone structure and remodelling pattern to that of humans (Borjesson & Peroni, 2011). The horse is already well established as an animal model for several musculoskeletal injuries like focal cartilage, tendon or ligament injuries, since many of these are similar in horses to those seen in human athletes (Koch et al., 2009). Moreover, several infectious, allergic/atopic, developmental, and autoimmune diseases have a similar pathogenic etiology between humans and domestic animals (Gershwin, 2007). Therefore, it can be concluded that horses could serve as an animal model for cell-based therapies to treat these human pathologies too.

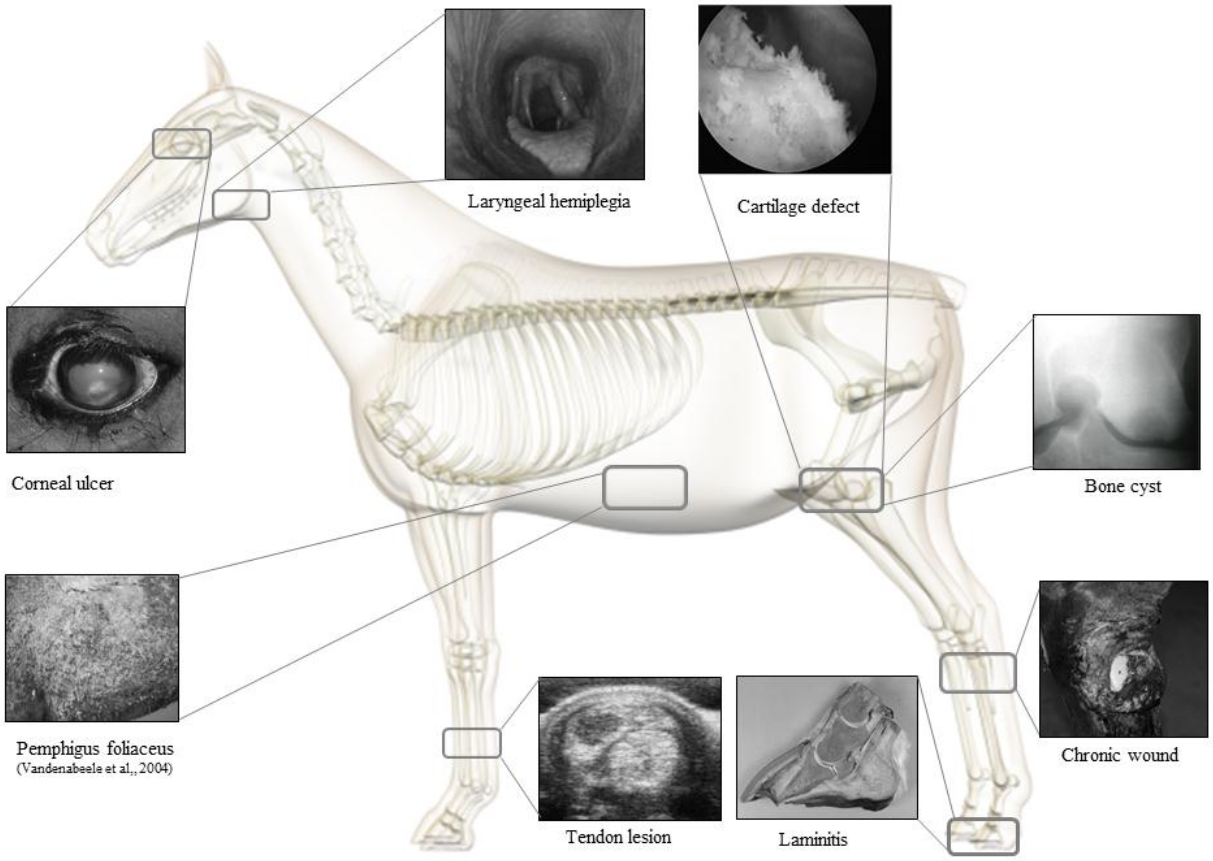

Figure 6. Schematic overview of possible applications of equine mesenchymal stromal cells in veterinary medicine.

a. Orthopedic injuries

The ability of MSC to differentiate into various tissues of mesodermal origin holds great potential for the repair and regeneration of tendon, cartilage, and bone (Taylor et al., 2007). An overview of clinical studies using equine MSC to treat musculoskeletal diseases in the horse, is given in Table 4. A positive therapeutic effect of MSC for the treatment of tendon lesions has been suggested before (Alves et al., 2011), and is also evident from the studies listed in Table 4.

Tendon

Injuries to the palmary situated tendons (i.e. superficial digital flexor tendon, deep digital flexor tendon and the suspensory ligament) are very common in competition horses subjected to high-intensity exercise due to the cumulative degenerative damage to the tendons which are already operating close to their functional limits (Richardson et al., 2007). In response to acute injury, there is an initial temporary inflammatory reaction characterized by oedema and pain (Richardson et al., 2007). During the healing process, the newly formed collagen of the scar tissue is less highly cross-linked and as such functionally deficient when compared to a normal tendon (Alves et al., 2011). Consequently, there is a substantial risk of reduced performance and/or re-injury (Godwin et al., 2012). Standard conservative treatment includes prolonged confinement and controlled exercise for up to 12 months after injury (Guest et al., 2008a). The poor success with conventional therapy further supports the need to search for novel treatments which should aim at restoring functionality and regenerating a tissue as close to the tendon as possible (Richardson et al., 2007). Frequently, the typical 'core lesion' in the superficial digital flexor tendon occurs centrally located within the tendon, is extended in length and still surrounded by intact tendon tissue (Brehm et al., 2012). As such, the equine MSC suspension can be administered into the lesion without the need for artificial scaffold material (Brehm et al., 2012). Intralesional administration of MSC in horses with tendinitis might stimulate the intrinsic healing, decrease the initial inflammatory reaction and scar tissue formation, and reduce the re-injury rate (Gutierrez-Nibeyro, 2011) (Fig.7).

Table 4. An overview of clinical studies using equine MSC to treat musculoskeletal diseases in the horse. Where applicable, the efficacy of the MSC treatment, i.e. effect as assessed by diagnostic tools or percentage (%) of horses re-injured after MSC treatment versus the control group, is indicated.

Study	N horses	Control group	Nature of the lesion	Diagnosis	Follow-up period	Efficacy MSC treatment vs control
Tendon						
Smith et al., 2003	1	NI	Natural	CE + U	6 weeks	NA
Pacini et al., 2007	11	Conservative treatment	Natural	CE + U	2 years	18.2% vs 100%
Del Bue et al., 2008	16	NI	Natural	CE + U	240 days	12.5% vs NI
Guest et al., 2008a	2	NI	Induced	IHC	10 and 34 days	No significant effect
Nixon et al., 2008	8	PBS	Induced	U + H + IHC+ PCR + BA	6 weeks	Positive effect
Smith et al., 2008	500	NI	Natural	CE + U	> 1 year	13-36%* vs NI
Ferris et al., 2009	97	NI	Natural	CE	7-39 months	15-27%
Leppänen et al., 2009	58	NI	Natural	PI	18-24 months	26.4% vs NI
Schnabel et al., 2009	12	Control limb: PBS	Induced	U + MT + H + PCR + BA	8 weeks	Positive effect
Crovace et al., 2010	6	Saline	Induced	CE + U + H + IHC	21 weeks	Positive effect
De Mattos Carvalho et al., 2011	8	Control limb: no MSC	Induced	CE+ U + H + IHC	8-21 weeks	Positive effect
Godwin et al., 2012	141	NI	Natural	CE + U + S + H	3 years	27.4% vs NI
Marfe et al., 2012a	3	Conservative treatment	Natural	U	3 years	0% vs 100%
Cartilage						
Wilke et al., 2007	6	Control limb: PBS	Induced	A + H + (I)HC + ISH	8 months	No significant effect
Ferris et al., 2009	40	NI	Natural	PI	21 months	28% vs NI
Frisbie et al., 2009	24	Placebo	Induced	CE + RX + H + punction	70 days	No significant effect
McIlwraith et al., 2011	10	Control limb: hyaluron alone	Induced	CE + RX + A + MRI + H + HM + IHC + BA	12 months	No significant effect

NI not included; CE clinical examination; U ultrasonography; NA not applicable; IHC immunohistochemistry; RX radiography; H histopathology; PCR polymerase chain reaction; BA biochemical analysis; PI phone interview; MT mechanical testing; S scintigraphy; A arthroscopy; ISH in situ hybridization; HM histomorphometry; MRI magnetic resonance imaging; *depending on discipline

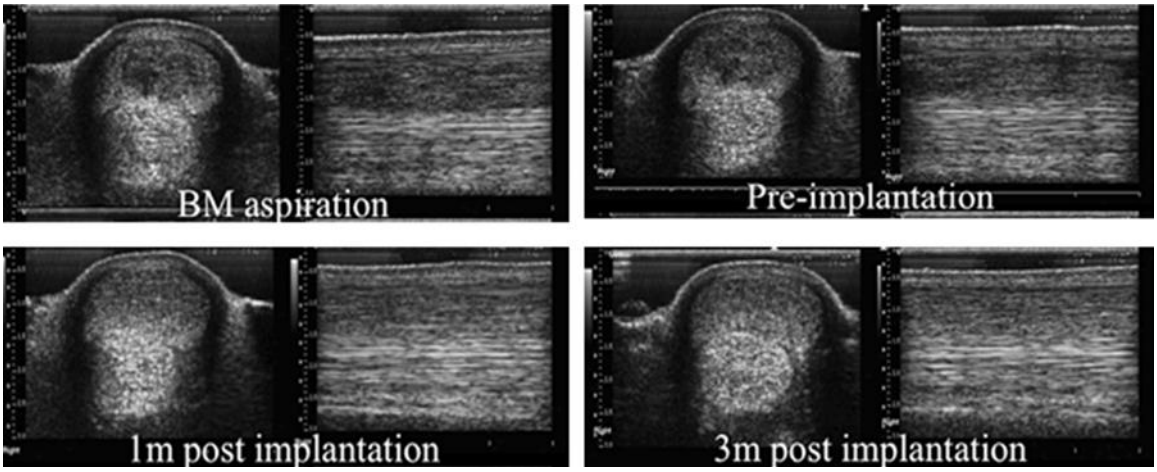

Figure 7. Ultrasonographic images of a superficial digital flexor tendon lesion on different follow-up time points (Godwin et al., 2012). The evolution of the lesion is described from the moment of BM aspiration and the subsequent MSC implantation (at 7 days) until 3 months later. The lesion is rapidly filled up while no apparent adverse effects are present.

Since the presence of mature fibrous tissue within the tendon would make MSC implantation more difficult and its persistence would reduce the benefits of the MSC therapy, chronic recurrent injuries are not considered ideal cases for stem cell therapy (Richardson et al., 2007). Some studies recommend to apply stem cell therapy within one month of injury, i.e. after the initial inflammatory phase but before fibrous scar tissue is formed (Richardson et al., 2007; Godwin et al., 2012). In a study of Leppänen et al. (2009), 60% of the horses suffering from superficial digital flexor tendinopathy or suspensory desmopathy and treated with AT-derived MSC, returned to full athletic function, while 26% were re-injured in the 18- to 24 month follow-up period. Nevertheless, 50% of the horses with recurrent injuries had previously injured the same tendon before MSC treatment (Lëppanen et al., 2009), confirming the previous statement that chronic recurrent injuries are not ideal.

In conclusion, a positive therapeutic effect of MSC for the treatment of tendon lesions has been suggested (Alves et al., 2011). In several studies in horses with induced tendinitis using collagenase, improved histological scores were demonstrated following MSC treatment when compared to saline-treated controls (Nixon et al., 2008; Schnabel et al., 2009). Regardless the

time of implantation, the re-injury rate has been shown to be significantly lower for horses treated with MSC (24%) in comparison to conservative therapy (56%) (Frisbie & Smith, 2010; Godwin et al., 2012). Also in naturally occurring incomplete core lesions of the superficial digital flexor tendon, 9 of 11 race horses treated with autologous BM-derived MSC in the lesion displayed a correct parallel orientation of the tendon fibers on ultrasound after 3 to 6 months, and returned to racing with good or even better results in their previous category of competition (Pacini et al., 2007). After a follow-up period of more than 2 years, all of them were still racing. In the control group treated by conservative therapy, most of the horses showed fibrosis during the healing process on ultrasound, and all of them were re-injured after 4 to 12 months. These results are supported by the clinical experiences of Burk and Brehm (2011): 84.5% of the 58 patients were back in sports or full training after MSC treatment without being re-injured after one year follow-up period.

Cartilage

Due to the hypocellular and avascular nature of articular cartilage, the ability to obtain effective repair is limited (Frisbie & Stewart, 2011). Full thickness cartilage defects in horses heal with fibrous tissue that might become fibrocartilage which has inferior biomechanical properties compared to articular hyaline cartilage (Taylor et al., 2007). Giving the ability of MSC to undergo chondrogenic differentiation, much of the recent research on cartilage resurfacing in the horse has focused on the use of MSC (Frisbie & Stewart, 2011). Although this differentiation has been demonstrated *in vitro*, the *in vivo* use of such cell-based therapy might be hampered by the compressive load exerted on the injected cells and scaffolds (Koch et al., 2009). In a controlled study of Wilke et al. (2007), equine BM-derived MSC were implanted in 15-mm cartilage defects which were surgically created in the lateral trochlear

ridge of the distal femur. The early healing response was improved as assessed at a 30-day arthroscopic evaluation but no significant differences were observed between the MSC-treated and the control group on the long-term (i.e. after 8 months).

The technique of 'microfracture' to provide access for chondrogenic progenitor cells and growth factors of the subchondral BM compartment into the base of the cartilage defect, has been applied in equine surgery in order to stimulate the cartilage repair (Taylor et al., 2007; Frisbie & Stewart, 2011). The outcome of this technique might be substantially improved when MSC are co-administered into the joint space (Frisbie & Stewart, 2011). In a recent study of McIlwraith et al. (2011), microfracture alone and microfracture in combination with the intra-articular administration of BM-derived MSC, were compared in order to test the ability of BM-derived MSC to enhance the healing response in full-cartilage defects. No significant clinical improvements or histological differences were observed although a statistically significant improvement in aggrecan content in the microfractured defects that received BM-derived MSC was seen. The latter could be beneficial to the durability and quality of the repair tissue and its ability to resist compression (McIlwraith et al., 2011). Still, the clinical importance of these findings awaits further confirmation.

The treatment of osteoarthritis either by conventional therapy or by cell-based therapy, is even more challenging than the repair of focal cartilage defects, since the articular cartilage damage in osteoarthritis is often more diffuse and not only both corresponding surfaces but also periarticular tissues such as the synovial membrane, the joint capsule, ligaments, menisci and subchondral bone can be impaired (Frisbie & Stewart, 2011). In a controlled study of Frisbie et al. (2009), both BM-derived MSC and AT-derived MSC were used in an established carpal osteochondral fragment model, in which osteoarthritis was induced by bone and cartilage debris with a negligible destabilization of the joint. A slight improvement in clinical signs and disease-modifying effects was noticed when using BM-derived MSC while

an up-regulation of proinflammatory cytokines in the synovial fluid concentrations was demonstrated when using AT-derived MSC (Frisbie et al., 2009). The use of MSC appears to be indicated in cases of loss of soft tissue structures leading to instability, for instance when meniscal damage is present (Frisbie & Smith, 2010). Therefore, a multicentre trial was carried out with moderately to severely affected patients of which the diagnosis was surgically confirmed and routine treatments failed (Ferris et al., 2009). Autologous BM-derived MSC were administered intra-articularly and cases were followed up for on average 21 months post-treatment (Ferris et al., 2009). Eleven of the 39 horses did not achieve any work status prior to follow-up while 38% returned to work at a lower level and 38% returned to their prior level of work or even exceeded this (Frisbie & Smith, 2010).

Bone

In contrast to tendon and cartilage repair, bone fractures usually regenerate with similar biochemical and biomechanical properties as the original tissue (Taylor et al., 2007). However, when large quantities of bone need to be regenerated, it might be required to stimulate the natural processes of bone repair (Kraus & Kirker-Head, 2006). Examples in which additional support might be necessary include substantial loss of host bone from trauma or tumor resection, arthrodesis, spinal fusion, non- or delayed unions, osseous cyst-like lesions, metabolic disease, arthroplasty or insufficient healing potential of the host because of local or systemic disease or old age (Kraus & Kirker-Head, 2006; Taylor et al., 2007). In veterinary orthopedics, many of the current techniques to aid fracture healing and stimulate bone formation involve the use of (autologous) bone grafts since these provide both osteogenic cells as well as osseous matrix (Milner et al., 2011). However, such an autologous graft must be harvested from another site which can result in donor-site morbidity (Vertenten

et al., 2009). Furthermore, the number of osteoprogenitor cells has been reported to vary between donor sites and might be less potent due to either an age-related decline in MSC number or a reduced metabolic function with increasing age (Koch et al., 2009). Allogeneic grafts, on the other hand, have a lower osteogenic capacity, a higher resorption rate, a larger immunogenic response, and less extensive revascularization, besides the risk of a possible viral contamination of the graft material (Vertenten et al., 2009).

In horses, a few preliminary experiments have been performed in which a pastern joint arthrodesis was supported by a combined therapy of stem cells and a bone replacement material, resulting in a good development of bone fusion (Brehm et al., 2012). However, no controlled clinical studies on the application of MSC in bone regeneration in horses have yet been reported.

b. Non-orthopedic injuries

Horses naturally acquire many diseases with shared pathophysiology compared to their human counterparts. For these diseases, MSC therapy might improve the current treatment options in horses. Besides, equines might serve as a valuable animal model for evaluating the potential of MSC therapy in human medicine as described above (Koch et al., 2009; Brehm et al., 2012). In the following paragraphs, a brief overview will be given on the potential of MSC for non-orthopedic injuries with emphasis on those diseases with a similar pathogenesis in both humans and horses.

Immune-mediated and inflammatory diseases

Mesenchymal stromal cells are known to modulate local inflammatory responses and recruit local autologous stem cells inside injured tissues to stimulate cell survival and tissue repair (Stewart & Stewart, 2011). Therefore, MSC can be useful in cases of organ-

transplantation, inflammatory and auto-immune diseases (Sensebe et al., 2009). Additionally, there is no evidence of systemic immunosuppression or increased risk of infections as possible side-effects when MSC are administered to immune-competent patients, suggesting that the immunomodulatory effects of MSC are restricted to inflamed tissues (Sensebe et al., 2009). A number of studies with animal models demonstrated the efficacy of MSC as a tool for immunomodulation in the protection against allograft rejection, autoimmune encephalomyelitis, collagen-induced arthritis, sepsis, systemic lupus erythematosus, and autoimmune myocarditis (Augello et al., 2005; Pluchino et al., 2005; Zappia et al., 2005; Einstein et al., 2007; Ohnishi et al., 2007; Gonzalez et al., 2009; Gonzalez-Rey et al., 2009; Pistoia et al., 2010; Sordi & Piemonti, 2011).

Some examples of equine autoimmune pathologies for which stem cell therapy can be of relevance include equine recurrent uveitis, an organ-specific, T-cell mediated autoimmune disease of high prevalence in horses (i.e.10%) (Deeg et al., 2008). High proportions of T cells infiltrate the inner eyes of horses suffering from recurrent uveitis and form lymphoid follicles in the iris stroma (Deeg et al., 2008). Pemphigus foliaceus is the most common autoimmune skin disease in horses. It is characterized by the presence of mAbs against cell-adhesion proteins on the cell membrane of keratinocytes, leading to clinical symptoms such as scaling and crusting (Vandenabeele et al., 2004). Equine systemic lupus erythematosus is a rare autoimmune disease caused by B cell hyperactivity which results in the production of mAbs against many tissues (Clark, 1988). The etiopathogenesis is largely unknown and a variety of symptoms are present such as synovitis, polyarthritis, glomerulonephritis, generalized dermatitis and edema of the extremities which is part of the generalized lymphadenopathy (Clark, 1988; Gershwin, 2005). Stem cell treatment could be relevant for these diseases regarding their autoimmune and inflammatory pathophysiological components.

Ischemic diseases

Stem cell therapy could also be used to treat ischemic diseases which cause oxygen deprivation, cell injury and related organ dysfunction (Chen et al., 2006). Although ischemic injuries are usually local in nature, they are often part of disorders with a highly complex pathophysiology which involves many biochemical changes in different cell types (Lange et al., 2005; Chen et al., 2006). The multi-differentiation and immunomodulatory abilities of MSC provide the opportunity of using them in the treatment of a variety of diseases such as stroke, ischemic retinopathy, myocardial infarction, ischemic diseases of the liver, ischemic renal failure, and ischemic limb dysfunction (Chen et al., 2006). Moreover, the functional recovery of the damaged tissue is supported by circulating stem cells since they appear to migrate specifically to ischemic regions (Chen et al., 2006). In a rat model where ischemic acute renal failure was induced, it was demonstrated that intravascular administration of BM-derived MSC enhanced the recovery of the renal function and revealed higher proliferative and lower apoptotic indexes (Lange et al., 2005).

In horses, laminitis is a multifactorial disease of the equine foot with various initiating causes including local ischemia (Engiles, 2010). Therefore, it has been proposed that stem cell therapy might improve the current treatment options (Koch et al., 2009). Perinatal asphyxia syndrome in foals usually arises from the combination of ischemia and hypoxemia and affects many organs (Galvin & Collins, 2004). The central nervous system may suffer the most profound damage, depending on the degree of hypoxia, but also gastrointestinal manifestations, adverse effects on the cardiovascular system, decrease in pulmonary perfusion, and damage to the renal tubular cells, are known clinical findings (Galvin & Collins, 2004). Based on the findings demonstrated in the rat model, equine MSC might support current therapeutic possibilities in horses.

Wound repair

It has been recently demonstrated in a mouse model that intravenously administered murine MSC at the site of a wound are able to accumulate and differentiate into multiple skin cell types including keratinocytes, endothelial cells and pericytes (Sasaki et al., 2008). In a preliminary study of Badiavas et al. (2003) on human patients, autologous BM-derived MSC were used to treat chronic wounds (i.e. older than 1 year) which were non-responsive to treatment. Complete closure and evidence of dermal rebuilding was observed in all 3 patients.

As horses are predisposed to traumatic wounds that can be labor intensive and expensive to manage, equine MSC could play an important role in wound repair considering their potential to improve the healing of skin defects (Sensebe et al., 2009; Theoret, 2009). In a retrospective study of 422 horses with traumatic wounds, primary closure was obtained in only 24% of the wounds (Wilmink et al., 2002). The historical gold standard to replace lost skin is an autologous skin graft but unfortunately, graft failure is relatively common in equine patients due to infection, inflammation, fluid accumulation beneath the graft, and motion (Theoret, 2009). Besides, full-thickness autografting is limited to relatively small wounds since the horse lacks redundant donor skin (Theoret, 2009). In equine veterinary medicine, only one study describes the use of MSC at the site of a surgically repaired soft palate defect (Carstanjen et al., 2006). Labeled autologous BM-derived MSC were implanted into the repaired defect at surgery and 14 days later, the horse was euthanized. Microscopic examination revealed that the MSC were oriented and integrated along the axis of the skeletal myocytes under the epithelium, which is indicative for a successful engraftment. Taken together, these preliminary data suggest that MSC indeed might contribute to wound healing.

Ophthalmology

A study of Ma et al. (2006) evaluated in a rat model whether or not MSC could be used to treat corneal disorders. For this purpose, human MSC were grown and expanded on amniotic membranes, and subsequently transplanted into corneas 7 days after chemical burns (Ma et al., 2006). The corneal surface was successfully reconstructed 4 weeks later although the underlying mechanism remained largely unknown. The therapeutic effect seemed associated with the inhibition of both inflammation and angiogenesis after MSC transplantation, rather than with the epithelial differentiation of MSC (Ma et al., 2006).

In veterinary ophthalmology, there is only one recent study describing the use of equine MSC in 4 chronic cases of corneal ulcer and one case of retinal detachment in horses, non-responsive to conventional treatment. All 4 patients showed significant improvement within 3 months (Marfe et al., 2012b).

Neurological disorders

As MSC can differentiate into neurogenic progenitors that express specific neuronal markers *in vitro*, the potential efficacy of MSC for functional repair of nervous tissues has been studied (Jamnig & Lepperdinger, 2012). In a study of Jung et al. (2009), a dog model was used in which spinal cord injuries were experimentally induced and subsequently treated with an intrathecal injection of BM-derived MSC. An improved neurological function in their pelvic limbs was detected when compared to control dogs. It was demonstrated that the exogenous transplanted canine MSC migrated towards the injured spinal cord lesion and provided a suitable environment for neuronal repair due the immunosuppressive, anti-inflammatory and trophic effects of these MSC (Jung et al., 2009).

Since neurodegenerative diseases display common pathological processes, a specific therapeutic agent like MSC could improve the symptoms of several neurodegenerative

disorders, based on their ability to replace damaged cells or secrete trophic factors and immunomodulating cytokines (Sadan et al., 2009). Equine myeloencephalopathy and equine motor neuron disease are examples of neurodegenerative disorders for which MSC therapy might be interesting. Also for laryngeal hemiplegia, caused by a progressive paralysis of the intrinsic laryngeal muscles (Kim & Xie, 2009), MSC might contribute to its treatment. However, it remains to be shown whether MSC display sufficient neurogenic differentiation capacity *in vivo* and whether they survive well after being transplanted (Jamnig & Lepperdinger, 2012).

c. Conclusions

In conclusion, the use of MSC in the treatment of equine injury has exciting potential and is expanding from its limited original application in orthopedic lesions into the treatment of ischemic, inflammatory, and neurologic disorders (Borjesson & Peroni, 2011). Nevertheless, sustained in-depth characterization of the MSC and well-designed prospective clinical trials remain mandatory in order to safe-guard optimal routine clinical use of these valuable equine MSC at the patients' benefit.

References

Alt EU, Senst C, Murthy SN, Slakey DP, Dupin CL, Chaffin AE, Kadowitz PJ, Izadpanah R. Aging alters tissue resident mesenchymal stem cell properties. Stem Cell Res 2012;8(2):215-225.

Alves AG, Stewart AA, Dudhia J, Kasashima Y, Goodship AE, Smith RK. Cell-based therapies for tendon and ligament injuries. Vet Clin North Am Equine Pract 2011;27(2):315-333.

Arnhold SJ, Goletz I, Klein H, Stumpf G, Beluche LA, Rohde C, Addicks K, Litzke LF. Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. Am J Vet Res 2007;68:1095-1105.

Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol 2005;35(5):1482-1490.

Badiavas EV, Falanga V. Treatment of chronic wounds with bone marrow-derived cells. Arch Dermatol 2003;139(4):510-516.

Baer PC, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. Stem Cells Int 2012;doi:10.1155/2012/812693.

Barreiro O, Yanez-Mo M, Serrador JM, Montaya MC, Vicente-Manzanares M, Tejedor R, Furthmayr H, Sanchez-Madrid F. Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. J Cell Biol 2002;157:1233-1245.

Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004;36:568-584.

Bartholomew S, Owens SD, Ferraro GL, Carrade DD, Lara DJ, librach FA, Borjesson DL, Galuppo LD. Collection of equine cord blood and placental tissues in 40 Thoroughbred mares. Equine Vet J 2009;41(8):724-728.

Berg L, Koch T, Heerkens T, Bessonov K, Thomsen P, Betts D. Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood. Vet Comp Orthop Traumatol 2009;22(5):363-370.

Borjesson DL, Peroni JF. The regenerative medicine laboratory: facilitating stem cell therapy for equine disease. Clin Lab Med 2011;31(1):109-123.

Boxall SA, Jones E. Markers for characterization of bone marrow multipotential stromal cells. Stem Celss Int 2012;doi 10.155/2012/975871.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005;122:947-956.

Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, Walliser U, Skutella T, Aicher WK. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. Am J Vet Res 2010;71:1228-1236.

Brehm W, Burk J, Delling U, Gittel C, Ribitsch I. Stem cell-based tissue engineering in veterinary orthopaedics. Cell Tissue Res 2012;347:677-688.

Brown VK, Ogle EW, Burkhardt AL, Rowley RB, Bolen JB, Justement LB. Multiple components of the B cell antigen receptor complex associate with the protein tyrosine phosphatase, CD45. J Biol Chem 1994;25:17238-17244.

Bunnell BA, Betancourt AM, Sullivan DE. New concepts on the immune modulation mediated by mesenchymal stem cells. Stem Cell Res Ther 2010;1(5):34.

Burk J, Brehm W. Stammzellentherapie von Sehnenverletzungen – klinische Ergebnisse von 98 Fällen. Pferdeheilkunde 2011;27:857-866.

Burk J, Ribitsch I, Gittel C, Juelke H, Kasper C, Staszyk C, Brehm W. Growth and differentiation characteristics of equine mesenchymal stromal cells derived from different sources. The Vet J 2012; in press.

Carrade DD, Affolter VK, Outerbridge CA, Walson JL, Galuppo LD, Buerchler S, Kumar V, Walker NJ, Borjesson DL. Intradermal injections of equine allogeneic umbilical cord-derived mesenchymal stem cells are well tolerated and do not elicit immediate or delayed hypersensitivity reactions. Cytotherapy 2011(a);13(10):1180-1192.

Carrade DD, Owens SD, Galuppo LD, Vidal MA, Ferraro GL, Librach F, Buerchler S, Friedman MS, Walker NJ, Borjesson DL. Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses. Cytotherapy 2011(b);13(4):419-430.

Carstanjen B, Desbois C, Hekmati M, Behr L. Succesful engraftment of cultured autologous mesenchymal stem cells in a surgically repaired soft palate defect in an adult horse. Can J Vet Res 2006;70(2):143-147.

Chen CP, Lee YJ, Chiu ST, Shyu WC, Lee MY, Huang SP, Li H. The application of stem cells in the treatment of ischemic diseaeses. Histol Histopathol 2006;21:1209-1216.

Clark EG. Saskatchewan. Equine systemic lupus erythematosus. Can Vet J 1988;29(7):595.

Clegg PD, Pinchbeck GL. Evidence-based medicine and stem cell therapy: how do we know such technologies are safe and efficacious? Vet Clin North Am Equine Pract 2011;27(2):373-382.

Cremonesi F, Violini S, Lange Consiglio A, Ramelli P, Ranzenigo G, Mariani P. Isolation, *in vitro* culture and characterization of foal umbilical cord stem cells at birth. Vet Res Commun 2008;32:139-142.

Crovace A, Lacitignola L, Rossi G, Francioso E. Histological and immunohistochemical evaluation of autologous cultured bone marrow mesenchymal stem cells and bone marrow mononucleated cells in collagenase-induced tendinitis of equine superficial digital flexor tendon. Vet Med Int 2010;doi:10.4061/2010/250978.

Colleoni S, Bottani E, Tessaro I, Mari G, Merlo B, Romagnoli N, Spadari A, Galli C, Lazzari G. Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. Vet Res Commun 2009;33:811-821.

Copland I, Sharma K, Lejeune L, Eliopoulos N, Stewart D, Liu P, Lachapelle K, Galipeau J. CD34 expression on murine marrow-derived mesenchymal stromal cells: impact on neovascularization. Exp Hematol 2008;36:93-103.

Csaki C, Matis U, Mobasheri A, Ye H, Shakibaei M. Chondrogenesis, osteogenesis and adipogenesis of canine mesenchymal stem cells: a biochemical, morphological and ultrastructural study. Histochem Cell Biol 2007;128:507-520.

Declercq HA, Verbeek RMH, De Ridder LIFJM, Schacht EH, Cornelissen MJ. Calcification as an indicator of osteoinductive capacity of biomaterials in osteoblastic cell cultures. Biomaterials 2005; 26:4964-4974.

Deeg CA, Hauck SM, Amann B, Pompetzki D, Altmann F, Raith A, Schmalzl T, Stangassinger M, Ueffing M. Equine recurrent uneïtis – a spontaneous horse model of uveitis. Opthalmic Res 2008;40:151-153.

Del Bue M, Ricco S, Ramoni R, Conti V, Gnudi G, Grolli S. Equine adipose-tissue derived mesenchymal stem cells and platelet concentrates: their association *in vitro* and *in vivo*. Vet Res Commun 2008;32:S51-S55.

de Mattos Carvalho A, Garcia Alves AL, Assis Golim M, Moroz A, Hussni CA, Galvao Gomes de Oliveira P, Deffune E. Isolation and immunophenotypic characterization of mesenchymal stem cells derived from equine adipose tissue. Vet Immunol Immunopathol 2009;132:303-306.

De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011;75:1431-1443.

Deuse T, Stubbendorff M, Tang-Quan K, Philips N, Kay MA, Eiermann T, Phan TT, Volk HD, Reichenspurner H, Robbins RC, Schrepfer S. Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells. Cell Transplant 2011;20(5):655-667.

Doarn R, Threlfall W, Kline R. Umbilical blood flow and the effects of premature severance in the neonatal horse. Theriogenol 1987;78:789-790.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.

Engiles JB. Pathology of the distal phalanx in equine laminitis: more than just a skin deep. Vet Clin North Am Equine Pract 2010:26(1):155-165.

Einstein O, Fainstein N, Vaknin I, Mizrachi-Kol R, Reihartz E, Grigoriadis N, Lavon I, Baniyash M, Lassman H, Ben-Hur T. Neural precursors attenuate autoimmune encephalomyelitis by peripheral immunosuppression. Ann Neurol 2007;61(3):209-218.

Ferris DJ, Frisbie DD, Kisiday JD, McIlwraith CW, Hague BA, Major MD, Schneider RK, Zubrod CJ, Watkins JJ, Kawcak CE, Goodrich LR. Clinical follow-up of horses treated with bone marrow-derived mesenchymal stem cells for musculoskeletal lesions. AAEP proceedings 2009;55:59-60.

Fortier LA. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005;34(5): 415-423.

Fortier LA, Travis AJ. Stem cells in veterinary medicine. Stem Cell Res Ther 2011;2:9.

Friedenstein AJ, Gorskaja UF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 1976;4:267-274.

Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM, McIlwraith CW. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. J Orthop Res 2009;27(12):1675-180.

Frisbie DD, Smith RK. Clinical update on the use of mesenchymal stem cells in equine orthopaedics. Equine Vet J 2010;42(1):86-89.

Frisbie DD, Stewart MC. Cell-based therapies for equine joint disease. Vet Clin North Am Equine Pract 2011;27(2):335-349.

Fukuda K. Molecular characterization of regenerated cardiomyocytes derived from adult mesenchymal stem cells. Congenit. Anom. 2002;42:1-9.

Galvin N, Collins D. Perinatal asphyxia syndrome in the foal: review and a case report. Irish Vet J 2004;57(12):707-714.

Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RCR. SSEA-4 identifies mesenchymal stem cells from bone marrow. Blood 2007;109:1743-1751.

Garbade J, Schubert A, Rastan AJ, Lenz D, Walther T, Gummert JF, Dhein S, Mohr F-W. Fusion of bone-marrow derived stem cells with cardiomyocytes in a heterologous *in vitro* model. Eur J Cardiothorac Surg 2005;28:685-691.

Gershwin LJ. Antinuclear antibodies in domestic animals. Ann NY Acad Sci 2005;1050:364-370.

Gershwin LJ. Veterinary autoimmunity-autoimmune diseases in domestic animals. Ann NY Acad Sci 2007;1109:109-116.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao M. Differences between human and mouse embryonic stem cells. Dev Biol 2004;269:360-380.

Giovannini S, Brehm W, Mainil-Varlet P, Nesic D. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. Differentiation 2008;76:118-129.

Godwin EE, Young NJ, Dudhia J, Beamish IC, Smith RK. Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. Equine Vet J 2012;44(1):25-32.

Gonzalez MA, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum 2009;60(4):1006-1019.

Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58(7):929-939.

Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 2003;4:117.

Guest DJ, Allen WR. Expression of cell-surface antigens and embryonic stem cell pluripotency genes in equine blastocysts. Stem Cells Dev 2007;16:789-795.

Guest DJ, Smith MR, Allen WR. Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: preliminary study. Equine Vet J 2008a;40(2):178-181.

Guest DJ, Ousey JC, Smith MRW. Defining the expression of marker genes in equine mesenchymal stromal cells. Stem Cells Cloning 2008b;1:1-9.

Gutierrez-Nibeyro SD. Commercial cell-based therapies for musculoskeletal injuries in horses. Vet Clin North Am Equine Pract. 2011;27(2):363-371.

Guweidhi A, Kleeff J, Adwan H, Giese NA, Wente MN, Giese T, Büchler MW, Berger MR, Friess H. Osteonectin influences growth and invasion of pancreatic cancer cells. Ann Surg 2005;242:224-234.

Hegewald AA, Ringe J, Bartel J, Krüger I, Notter M, Barnewitz D, Kaps C, Sittinger M. Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. Tissue Cell 2004;36:431-438.

Hill CE, Proschel C, Noble M, Mayer-Proschel M, Gensel JC, Beattie MS, Bresnahan JC. Acute transplantation of glial-restricted precursor cells into spinal cord contusion lesions: survival, differentiation, and effects on lesion environment and axonal regeneration. Exp Neurol 2004;190:289-310.

Hoogduijn MJ, Popp F, Verbeek R, Masoodi M, Nicolaou A, Baan C, Dahlke MH. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. Int Immunopharmacol 2010;10(12):1496-1500.

Horvath G, Serru V, Clay D, Billard M, Boucheix C, Rubinstein E. CD19 is linked to the integrin-associated tetraspans CD9, CD81 and CD82. J Biol Chem 1998;273:30537-30543.

Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy Position Statement. Cytotherapy 2005;7:393-395.

Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T, Mitchell KE. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem Biophys Res Commun 2007;362:347-353.

Ibrahim S, Saunders K, Kydd JH, Lunn DP, Steinbach F. Screening of anti-human leukocyte monoclonal antibodies for reactivity with equine leukocytes. Vet Immunol Immunopathol 2007;119:63-80.

Jamnig A, Lepperdinger G. From tendon to nerve: an MSC for all seasons. Can J Physiol Pharmacol 2012;90(3):295-306.

Jiang Y, Jahagirdar BN, Reinhardt R, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-49.

Jung D-I, Ha J, Wang B-T, Kim J-W, Quan F-S, Lee J-H, Woo E-J, Park H-M. A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury. J Neurolog Sciences 2009;285:67-77.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294-1301.

Kim MS, Xie H. Use of electroacupuncture to treat laryngeal hemiplegia in horses. Vet Rec 2009;165(20):602-604.

Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, Deans RJ, McIntosh KR. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci 2005;12(1):47-57.

Knothe Tate ML. Top down and bottom up engineering of bone. J Biomech 2011;44:304-312.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

Koch TG, Berg LC, Betts DH. Concepts for the clinical use of stem cells in equine medicine. Can Vet J 2008;49:1009-1017.

Koch TG, Berg LC, Betts DH. Current and future regenerative medicine – principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine. Can Vet J 2009;50:155-165.

Kode JA, Mukherjee S, Joglekar MV, Hardikar AA. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy 2009;11(4):377-391.

Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP. Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. Stem Cells 2006;24:1613-1619.

Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther 2007;9(1):204.

Kogler G, Sensken S, Airey JA, Trapp T, Müschen M, Feldhahn N, Liedke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Müller HW, Zanjani E, Wernet P. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 2004; 200(2):123-135.

Kraus KH, Kirker-Head C. Mesenchymal stem cells and bone regeneration. Vet Surg 2006;35(3):232-242.

Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005;19:29-38.

Lange C, Tögel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, Westenfelder C. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney Int 2005;68:1613-1617.

Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S, Parolini O, Cremonesi F. Characterization and potential applications of progenitor-like cells isolated from horse amniotic membrane. J Tissue Eng Regen Med 2011;doi:10.1002/term.565.

Latchman DS. Transcription factors: an overview. Int J Biochem Cell Biol 1997;29:1305-1312.

Lee OK, Kuo TK, Chen W, Lee K, Hsieh S, Chen T. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669-1675.

Leppänen M, Tulamo R-M, Heikkilä P, Katiskalahti T. Follow-up of equine tendon and ligament injuries 18-24 months after treatment with enriched autologous adipose-derived mesenchymal stem cells – a clinical study. Regen Med 2009;4(6):S21-S22.

Li G, Zhang X, Wang H, Wang X, Meng C-H, Chan C-Y, tai Wai Yew D, Tsang KS, Li K, Tsai S-N, Ngai S-M, Chao Han Z, Lin MC-M, He M-L, Kung H-F. Comparative proteomic analysis of mesenchymal stem cells derived from human bone marrow, umbilical cord, and placenta: implication in the migration. Proteomics 2009;9:20-30.

Li XIHE, Zhou SG, Imreh MP, Ährlund-Richter L, Allen WR. Horse embryonic stem cell lines from the proliferation of inner cell mass cells. Stem Cells Dev 2006;15:523-531.

Lunn DP. A comparative review of human and equine leucocyte differentiation antigens. Br Vet J 1993;149:31-49.

Ma Y, Xu Y, Xiao Z, Yang W, Zhang C, Song E, Du Y, Li L. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. Stem Cells 2006;24:315-321.

Marfe G, Rotta G, De Martino L, Tafani M, Fiorito F, Di Stefano C, Polettini M, Ranalli M, Russo MA, Gambacurta A. A new clinical approach: use of blood-derived stem cells (BDSCs) for superficial digital flexor tendon injuries in horses. Life Sci 2012a;90:825-830.

Marfe G, Massaro-Giordano M, Ranalli M, Cozzoli E, Di Stefano C, Malafoglia V, Polettini M, Gambacurta A. Blood derived stem cells: an ameliorative therapy in veterinary ophthalmology. J Cell Physiol 2012b;227(3):1250-1256.

Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M. Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood. Tissue Eng Part C 2010;16:771-781.

McBride SH, Knothe Tate ML. Modulation of stem cell shape and fate, B: mechanical modulation of cell shape and gene expression. Tissue Eng 2008;14:1573-1580.

McGuckin CP, Forraz N, Baradez M-O, Navran S, Zhao J, Urban R, Tilton R, Denner L. Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif 2005;38:245-255.

McIlwraith CW, Frisbie DD, Rodkey WG, Kisiday JD, Werpy NM, Kawcak CE, Steadman JR. Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. Arthroscopy 2011;27(11):1552-1561.

Milner PI, Clegg PD, Stewart MC. Stem cell-based therapies for bone repair. Vet Clin North Am Equine Pract 2011;27(2):299-314.

Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, Halvorsen YD, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells 2006;24:376-385.

Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 2006;441:1068-1074.

Nichol JW, Khademhosseini A. Modular tissue engineering: engineering biological tissues from the bottom up. Soft Matter 2009;5(7):1312-1319.

Nixon AJ, Dahlgren LA, Haupt JL, Yeager AE, Ward DL. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. Am J Vet Res 2008;69(7):928-937.

Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M, Kodama M, Ishibashi-Ueda H, Kangawa K, Kitamura S, Nagaya N. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol 2007;42(1):88-97.

Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehringer G, Bornhäuser M, Werner C. Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. Stem Cells 2004;22:377-384.

Pacini S, Spinabella S, Trombi L, Fazzi R, Galimberti S, Dini F, Carlucci F, Petrini M. Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses. Tissue Eng 2007;13:2949-2955.

Passeri S, Nocchi F, Lamanna R, Lapi S, Miragliotta V, Giannessi E, Abramo F, Stornelli MR, Matarazzo M, Plenteda D, Urciuoli P, Scatena F, Coli A. Isolation and expansion of equine umbilical cord-derived matrix cells. Cell Biol Int 2009;33:100-105.

Peroni JF, Borjesson DL. Anti-inflammatory and immunomodulatory activities of stem cells. Vet Clin North Am Equine Pract 2011;27(2):351-362.

Pistoia V, Raffaghello L. Potential of mesenchymal stem cells for the therapy of autoimmune diseases. Expert Rev Clin Immunol 2010;6(2):211-218.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.

Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Corni G, Constantin G, Martino G. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature 2005;436(7048):266-271.

Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol 2009;39(12):3436-3446.

Quintavalla J, Uziel-Fusi S, Yin J, Boehnlein E, Pastor G, Blancuzzi V, Singh HN, Kraus KH, O'Byrne E, Pellas TC. Fluorescently labeled mesenchymal stem cells (MSCs) maintain multilineage potential and can be detected following implantation into articular cartilage defects. Biomaterials 2002;23(1):109-119.

Quirici N, Scavullo C, de Girolamo L, Lopa S, Arrigoni E, Lambertenghi Deliliers G, Brini AT. Anti-L-NFGR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. Stem Cells Dev 2010;19:915-925.

Radcliffe CH, Flaminio MJBF, Fortier LA. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev 2010;19:269-281.

Rastan AJ, Walther T, Kostelka M, Garbade J, Schubert A, Stein A, Dhein S, Mohr FW. Morphological, electrophysiological and coupling characteristics of bone marrow-derived mononuclear cells – an *in vitro* model. Eur J Cardiothorac Surg 2004;78:1989-1993.

Reed SA, Johnson SE. Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types. J Cell Physiol 2008;215:329-336.

Rege TA, Hagood JS. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer and fibrosis. The FASEB journal 2006;20:1045-1054.

Resta R, Thompson LF. T cell signaling through CD73. Cell Signal 1997;9:131-139.

Richardson LE, Dudhia J, Clegg PD, Smith R. Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury. Trends Biotechnol 2007;25:409-416.

Rozemuller H, Prins H-J, Naaijkens B, Staal J, Bühring H-J, Martens AC. Prospective isolation of mesenchymal stem cells from multiple mammalian species using cross-reacting anti-human monoclonal antibodies. Stem Cells Dev 2010;19(12):1911-1921.

Sadan O, Melamed E, Offen D. Bone marrow-derived mesenchymal stem cell therapy for neurodegenerative diseases. Expert Opin Biol Ther 2009;9(12):1487-1497.

Sanz-Rodriguez F, Guerrero-Esteo M, Botella L-M, Banville D, Vary CPH, Bernabeu C. Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the Lim family of proteins. J Biol Chem 2004;279:32858-32866.

Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell types. J Immunol 2008;180:2581-2587.

Schnabel LV, Lynch ME, van der Meulen MC, Yeager AE, Kornatowski MA, Nixon AJ. Mesenchymal stem cells and insulin-like growth factor-I gene-enhanced mesenchymal stem cells improve structural aspects of healing in equine flexor digitorum superficialis tendons. J Orthop Res 2009;27(10):1392-1398.

Sensebe L, Bourin P. Mesenchymal stem cells for therapeutic purposes. Transplantation 2009;87:S49-S53.

Shuh EM, Friedman MS, Carrade DD, Li J, Heeke D, Oyserman SM, Galuppo LD, Lara DJ, Walker NJ, Ferraro GL, Owens SD, Borjesson DL. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical-cord blood. Am J Vet Res 2009;70:1526-1535.

Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody Stro-1. Blood 1991a;78:55-62.

Simmons PJ, Torok-Storb B. CD34 expression by stromal precursors in normal adult human bone marrow. Blood 1991b;78:2848-2853.

Smith RKW, Korda M, Blunn GW, Goodship AE. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. Equine Vet J 2003;35:99-102.

Smith RKW. Mesenchymal stem cell therapy for equine tendinopathy. Disabil Rehabil 2008;30:1752-1758.

Sordi V, Piemonti L. Therapeutic plasticity of stem cells and allograft tolerance. Cytotherapy 2011;13:647-660.

Stewart MC. Cell-based therapies: current issues and future directions. Vet Clin North Am Equine Pract 2011;27(2):393-399.

Stewart MC, Stewart AA. Mesenchymal stem cells: characteristics, sources, mechanisms of action. Vet Clin North Am Equine Pract 2011;27(2):243-261.

Sun B, Roh K-H, Lee S-R, Lee Y-S, Kang K-S. Induction of human umbilical cord blood-derived stem cells with embryonic stem cell phenotypes into insulin producing islet-like structure. Biochem Biophys Res Commun 2007;354:919-923.

Taylor SE, Clegg PD. Collection and propagation methods for mesenchymal stromal cells. Vet Clin North Am Equine Pract 2011;27(2):263-274.

Taylor SE, Smith RKW, Clegg PD. Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet J 2007;39:172-180.

Theoret C. Tissue engineering in wound repair: the three “R”s-repair, replace, regenerate. Vet Surg 2009;38(8):905-913.

Tolar J, Le Blanc K, Keating A, Blazar BR. Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells 2010;28(8):1446-1455.

Toupadakis CA, Wong A, Genetos DC, Cheung WK, Borjesson DL, Ferraro GL, Galuppo LD, Leach JK, Owens SD, Yellowley CE. Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res 2010;71(10):1237-1245.

Ulloa-Montoya F, Verfaillie CM, Hu W-S. Culture systems for pluripotent stem cells. J Biosci Bioeng 2005;100:12-27.

Vandenabeele SIJ, White SD, Affolter VK, Kass PH, Ihrke P. Pemphigus foliaceus in the horse: a retrospective study of 20 cases. Vet Dermatol 2004;15:381-388.

Verfaillie CM, Pera MF, Lansdorp PM. Stem cells: hype and reality. Hematology 2002;369-391.

Vertenten G, Lippens E, Girones J, Gorski T, Declercq H, Saunders J, Van den Broeck W, Chiers K, Duchateau L, Schacht E, Cornelissen M, Gasthuys F, Vlaminck L. Evaluation of an injectable, photopolymerizable, and three-dimensional scaffold based on methacrylate-endcapped poly(D,L-lactideco-epsilon-caprolactone) combined with autologous mesenchymal stem cells in a goat tibial unicortial defect model. Tissue Eng Part A 2009;15(7):1501-1511.

Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 2006;35:601-610.

Vidal MA, Kilroy GE, Lopez MJ, Johnson JR, Moore RM, Gimble JM. Characterization of equine adipose tissue-derived stromal cells: adipogenic and osteogenic capacity and comparison with bone marrow-derived mesenchymal stromal cells. Vet Surg 2007;36(7):613-622.

Vidal MA, Robinson SO, Lopez MJ, Paulsen DB, Borkhsenious O, Johnson JR, Moore RM, Gimble JM. Comparison of chondrogenic potential in equine mesenchymal stromal cells derived from adipose tissue and bone marrow. Vet Surg 2008;37(8):713-724.

Vidal MA, Walker NJ, Napoli E, Borjesson DL. Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue. Stem Cells Dev 2012;21(2):273-283.

Violini S, Ramelli P, Pisani LF, Gorni C, Mariani P. Horse bone marrow mesenchymal stem cells express embryo stem cell markers and show the ability for tenogenic differentiation by *in vitro* exposure to BMP-12. BMC Cell Biol 2009;10:29.

Wang H-S, Hung S-C, Peng S-T, Huang C-C, Wei H-M, Guo Y-J, Fu Y-S, Lai M-C, Chen C-C. Mesenchymal stem cells in the Wharton's Jelly of the human umbilical cord. Stem Cells 2004;22:1330-1337.

Wilke MM, Nydam DV, Nixon AJ. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. J Orthop Res 2007;25(7):913-925.

Wilmink JM, van Herten J, van Weeren PR, Barneveld A. Retrospective study of primary intention healing and sequestrum formation in horses compared to ponies under clinical circumstances. Equine Vet J 2002;34(3):270-273.

Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res 2011;109(8):923-940.

Xu J, Liao WB, Gu DS, Liang L, Liu M, Du WT, Liu PX, Zhang L, Lu SH, Dong CL, Zhou B, Han Z. Neural ganglioside GD2 identifies a subpopulation of mesenchymal stem cells in umbilical cord. Cell Physiol Biochem 2009;23:415-424.

Yagi H, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML. Mesenchymal stem cells: mechanisms of immunomodulation and homing. Cell Transplant 2010;19(6):667-679.

Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 2005;106(5):1755-1761.

Zhao Y, Wang H, Mazzone T. Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. Exp Cell Res 2006;312:2454-2464.

Zola H, Swart B, Nicholson I, Aasted B, Bensussan A, Boumsell L, Buckey C, Clark G, Drbal K, Engel P, Hart D, Horejsi V, Isacke C, Macardle P, Malavasi F, Mason D, Olive D, Saalmueller A, Schlossman SF, Schwartz-Albiez R, Simmons P, Tedder TF, Uguccioni M, Warren H. CD molecules 2005: human cell differentiation molecules. Immunobiology 2005;106:3123-3126.

Zuk P. The intracellular distribution of the ES cell totipotent markers Oct4 and Sox-2 in adult stem cells differs dramatically according to commercial antibody used. J Cell Biochem 2009;106:867-877.

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2000;2:477-488.

CHAPTER 2

AIMS

The clinical applications of mesenchymal stromal cells (MSC) in equine veterinary medicine have been increasingly explored the past few years, while the research lags behind and important fundamental questions remain unanswered. More specifically, little or no standardization for the isolation and characterization of equine MSC was available, which is in sharp contrast to the detailed guidelines described for the unequivocal characterization of human MSC.

The general aim of this doctoral thesis was to gain a better fundamental insight into equine MSC. To this end, we used umbilical cord blood as a readily available source for the isolation of these equine cells.

The first aim of this thesis was to optimize the isolation protocol for umbilical cord blood-derived MSC (**Chapter 3**) and subsequently, to establish a uniform characterization of these putative equine MSC, a prerequisite for the proper interpretation and comparison of results from different research groups (**Chapter 4**).

In addition, it was investigated whether or not it is possible to cryopreserve the isolated mononuclear cells immediately after isolation, as such a strategy would avoid the use of a time-consuming work-up protocol to start the MSC cultures. The MSC could then be cultured from cryopreserved mononuclear cells when needed (**Chapter 5**).

Although bone marrow is the best known source for isolating MSC, the harvest of these cells is a highly invasive procedure. Therefore, different features of equine MSC isolated from three minimal-invasive sources, i.e. peripheral blood, umbilical cord matrix and umbilical cord blood, were compared to evaluate whether or not MSC isolated from these three sources have the same culture characteristics, differentiation capacities and display the same set of markers as MSC derived from bone marrow (**Chapter 6**).

CHAPTER 3

OPTIMIZATION OF THE ISOLATION AND CULTURE OF EQUINE MESENCHYMAL STROMAL CELLS ISOLATED FROM UMBILICAL CORD BLOOD

De Schauwer C, Meyer E, Cornillie P, De Vliegher S, Van de Walle GR, Hoogewijs MK, Declercq H, Govaere J, Demeyere K, Cornelissen M, Van Soom A. Optimization of the isolation, culture and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng Part C 2011; 17(11):1061-70.

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3.1. Abstract

Mesenchymal stromal cells (MSC) represent a promising population for supporting new clinical concepts in cellular therapy. A wide diversity of isolation procedures for MSC from umbilical cord blood (UCB) has been described for humans. In contrast, few data are available in horses. In the present study, a sedimentation method using hydroxyethyl starch (HES) and a method based on the lysis of red blood cells (RBC) using ammonium chloride (NH_4Cl) were compared with two density gradient separation methods (Ficoll-Paque and Percoll). Adherent cell colonies could be established using all four isolation methods. The mononuclear cell (MNC) recovery after Percoll separation, however, resulted in significantly more putative MSC colonies and therefore, this isolation method was used for all further experiments. Culture conditions such as cell density and medium or serum coating of the wells did not significantly affect putative MSC recovery. Isolated MSC using Percoll were subsequently differentiated towards the osteogenic, chondrogenic and adipogenic lineage. In addition, MSC were phenotyped by multicolor flow cytometry based on their expression of different cell protein markers. Cultured MSC were CD29, CD44 and CD90-positive and CD79α, macrophage/monocyte and MHC II-negative. In conclusion, this study reports optimized protocols to isolate, culture and characterize solid equine MSC from UCB.

3.2. Introduction

Stem cell therapy has recently received much attention in equine veterinary medicine. This interest is mainly raised by the fact that the musculoskeletal system represents a major part of the horse's value in sports, breeding and leisure activities (Koerner et al., 2006). Treatment with MSC could be revolutionary in equine orthopedic disease for those injuries where natural repair mechanisms do not deliver functional recovery or where current therapeutic strategies have minimal effectiveness (Richardson et al., 2007). These MSC are an attractive source of

multipotent stem cells which support hematopoiesis (Bieback et al., 2004) and can differentiate into different cell lines including adipocytes, osteocytes, chondrocytes, myocytes, astrocytes, and tenocytes (Covas et al., 2003). To date, equine MSC used for clinical therapy have mainly been isolated either from bone marrow (BM) or from adipose tissue (AT) (Smith et al., 2003; Leppänen et al., 2009). Although BM-derived MSC are most commonly used, MSC have also been isolated from other tissues including trabecular bone, brain, muscle, peripheral blood, tendon, synovial membrane, artery wall, umbilical cord matrix and UCB (Panepucci et al., 2004; Koerner et al., 2006; Hoynowski et al., 2007; Giovannini et al., 2008; Martinello et al., 2010). Most of these procedures, however, require invasive techniques to obtain sufficient numbers of MSC for clinical therapy and consequently, could lead to possible complications for the patient. On the other hand, UCB, which is normally discarded, can be readily collected without discomfort or danger to mother or neonate (Harris et al., 1994), and as such represents a valuable alternative source of MSC.

Several UCB fractionation procedures for the isolation of human MSC have been proposed based on the partial or complete removal of RBC and plasma (Regidor et al., 1999). These include (i) the use of sedimentation agents such as HES (Rubinstein et al., 1995; M-Reboredo et al., 2000), poligeline (Almici et al., 1996) or gelatin (Bertolini et al., 1995; Quillen & Berkman, 1996), (ii) simple manual or partially automated centrifugation (Ademokum et al., 1997) and (iii) density gradient cell separation protocols based on either Percoll (Newton et al., 1993; Barry & Murphy, 2004) or Ficoll (Campos et al., 1995; Colter et al., 2000; Bieback et al., 2004; Lee et al., 2004; Kern et al., 2006; Meyer et al., 2006; Vidal et al., 2006). In human medicine, few reports compare the efficacy of different isolation methods in terms of cell recovery (Almici et al., 1995; Almici et al., 1996; Quillen & Berkman, 1996; Regidor et al., 1999). In veterinary medicine, two studies have been published to date comparing

different isolation protocols for equine MSC which were derived from equine BM (Bourzac et al., 2010) or from UCB (Koch et al., 2009), respectively.

The major aim of this study was to compare four isolation methods to acquire putative equine MSC from UCB: one method based on the lysis of RBC using NH_4Cl, one sedimentation method with HES and two methods based on the use of density gradient separation i.e. Percoll and Ficoll-Paque. In addition, the importance of culture conditions such as cell density, culture medium and serum coating of the wells for *in vitro* culturing of putative UCB-derived equine MSC was evaluated. A final objective was to characterize the putative isolated MSC both by assessment of their differentiation capacities and by immunophenotyping.

3.3. Materials and methods

3.3.1. Umbilical cord blood collection

Umbilical cord blood was collected from full-term born foals immediately after birth, before the umbilical cord ruptured spontaneously. After clamping and disinfecting the umbilical cord with 70% alcohol, the umbilical vein was punctured and UCB was drained by gravity into a sterile standard 350-mL blood donor bag containing 49 mL CPD A anticoagulant (Terumo®), and subsequently stored at 4°C. A number of critical conditions, as described by Bieback et al. (2004), were included to decide whether further processing of the UCB was attempted. As such, the volume of all samples processed was more than 100 mL (197.1 ± 39.0 mL), storage time was less than 15 h (9.8 ± 3.0 h) and none of the samples showed signs of coagulation or hemolysis.

3.3.2. Cell isolation methods

Eight UCB samples were collected in order to compare the four different isolation methods. To fit the experimental design (see below), 2.8×10^7 isolated cells were required. To this end, different volumes of UCB were processed for each isolation method, i.e. 4 mL for NH_4Cl, 60 mL for Percoll, 120 mL for Ficoll-Paque, and 40 mL for HES.

a. Lysis of the RBC with NH_4Cl lysing solution

Fourteen mL NH_4Cl lysing solution (consisting of 8.9 g NH_4CL, 1.0 g $KHCO_3$, 37.0 mg tetrasodium EDTA in 1 L distilled water, all from Sigma, pH= 7.3) was added to a 15 mL Falcon tube containing 1 mL UCB at room temperature (RT). During 3 to 5 min, four tubes were inverted several times and subsequently centrifuged for 5 min at 300 ×g at RT. After removing the supernatant, the cell pellet was washed in Hank's balanced salt solution without Ca/Mg (HBSS) (Invitrogen) by centrifuging 5 min at 300 ×g, and finally resuspended in 5 mL HBSS.

b. Density gradient separation with Percoll

After centrifuging 60 mL UCB at 1000 ×g for 20 min at RT, the buffy coat fraction was collected and diluted 1:1 (v:v) with HBSS. Subsequently, the cell suspension was gently layered on an equal volume of Percoll (density 1.080 g/mL; GE Healthcare) and centrifuged for 15 min at 600 ×g at RT, as previously described (19). The interphase was collected, washed three times with HBSS by centrifuging 10 min at 200 ×g, and finally resuspended in 5 mL HBSS.

c. Density gradient separation with Ficoll-Paque

The MNC fraction was isolated by loading 30 mL of UCB onto 10 mL Ficoll-Paque PREMIUM® (GE Healthcare) in 50 mL Falcon tubes, as described by Koch et al. (2007). Four tubes were centrifuged for 30 min at 450 ×g at RT. After aspirating the supernatant, the

interphase was collected and washed twice with HBSS by centrifuging 5 min at 450 ×g, and finally resuspended in 5 mL HBSS.

d. Hydroxyethyl starch separation

Hydroxyethyl starch solution (HES 6%®; Braun) was added to UCB in a ratio of 1:5 (v:v). After centrifugation of four tubes for 5 min at 50 ×g at RT, the leukocyte-rich plasma, containing some RBC, was collected. This fraction was centrifuged again for 15 min at 600 ×g at RT. The supernatant was subsequently removed and the cell pellet was washed with HBSS by centrifuging 10 min at 200 ×g, and finally resuspended in 5 mL HBSS.

3.3.3. Media

Two different culture media were compared for the isolation and expansion of MSC from UCB. MesenCult® is a commercial MSC medium based on McCoy's medium (StemCell Technologies). The other culture medium used was largely based on the medium described by Koch et al. (2007), hereafter designated Koch's medium, and contains low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 30% fetal calf serum (FCS) (GIBCO), 10^{-7} M low dexamethazone, 50 µg/mL gentamycine, 10 µl/mL antibiotic antimycotic solution, 250 ng/mL fungizone (all from Sigma) and 2 mM ultraglutamine (Invitrogen). Expansion medium was identical to Koch's culture medium but without dexamethasone.

For the differentiation experiments, following media were used: (i) osteogenic medium, containing DMEM-LG (Invitrogen), 10% FCS (GIBCO), 0.2 mM L-ascorbic acid-2-phosphate (Fluka), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/mL gentamycine and 10 µl/mL antibiotic antimycotic solution (all from Sigma); (ii) chondrogenic medium based on the basal differentiation medium (Lonza), complemented with 10 ng/mL Transforming Growth Factor β3 (Sigma) and (iii) adipogenic induction medium containing

DMEM-LG (Invitrogen), 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL rh-insuline, 0.2 mM indomethacin, 15% rabbit serum, 50 µg/mL gentamycine and 10 µl/mL antibiotic antimycotic solution (all from Sigma); (iv) adipogenic maintenance medium which was identical to the adipogenic induction medium except for the omission of dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine.

3.3.4. Experimental design

After isolation, cells were seeded at three different cell concentrations (1×10^6, 2×10^6 and 4×10^6 cells/mL) in two different media (Mesencult and Koch's medium) using 2 mL medium per well. Moreover, isolated cells were seeded on uncoated polystyrene 6-well culture dishes (BD) as well as on polystyrene 6-well culture dishes coated with 100% FCS (GIBCO). As a result, UCB-derived blood cells from one mare were isolated using four different methods and 12 different culture conditions were evaluated for each isolation method. So, each UCB sample was subdivided in 48 units with one unit representing one tested condition (Fig.1).

3.3.5. Culture parameters and conditions

Cell viability was determined by trypan blue exclusion using the improved Neubauer hemocytometer, as previously described (Strober et al., 2001). Isolated cells were incubated at 38.5°C in a humidified atmosphere containing 5% CO_2. After overnight incubation, non-adherent cells were removed and fresh medium was added to the wells. The remaining non-adherent cells were removed by exchanging the culture medium every seven days. Cultures were inspected every three days for the formation of adherent spindle-shaped fibroblastoid cell colonies. Cells were passaged as soon as confluency exceeded 80% using 0.083% trypsin-EDTA (Sigma). The replating ratio after chemical cell detachment was 1:3.

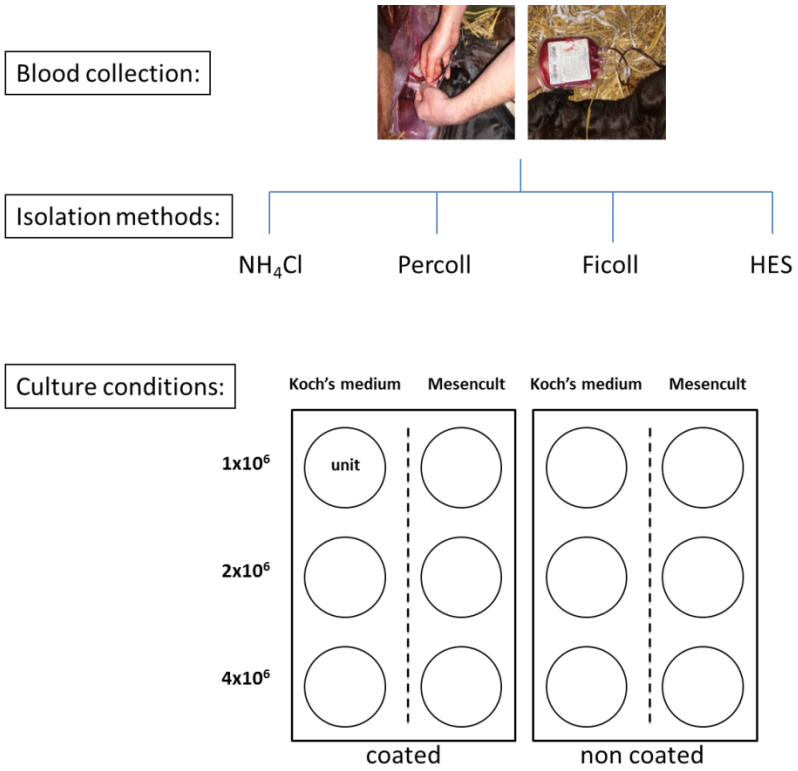

Figure 1. Schematic overview of the experimental design. For each UCB sample, blood cells were isolated using four different methods and for each isolation, 12 different culture conditions were tested such as culture medium, seeding concentration and coated versus non-coated wells. Each condition is represented by one unit.

3.3.6. Tri-lineage cell differentiation

Mononuclear cells from seventeen UCB samples were isolated using Percoll and cultured at a concentration of 4×10^6 cells/mL in uncoated T-25 culture flasks using Koch's medium. The medium was exchanged every 3-4 days. Cells were subsequently expanded in expansion medium for the next two passages. All experiments were performed in triplicates and non-induced cells in expansion medium were used as negative controls.

a. Osteogenic differentiation

The osteogenic differentiation was performed in six-well culture dishes with approximately 3000 cells/cm^2 which were cultured in expansion medium until 90-100% confluency was reached. Hereafter, osteogenic differentiation was induced with osteogenic medium which was exchanged every 3-4 days. Osteogenic differentiation was evaluated after 20 days of

culture using the Alizarine Red S and the Von Kossa histological staining after fixation with 4% buffered formaldehyde, as well as by detecting alkaline phosphatase activity (Millipore®, Alkaline Phosphatase Detection kit), according to the manufacturer's instructions.

b. *Chondrogenic differentiation*

Approximately 2.5×10^5 cells were centrifuged in 15-mL conical Falcon tubes (150 ×g for 5 min at RT), whereafter 0.5 mL chondrogenic medium was added to each tube without disturbing the cell pellet. These micromass culture systems were maintained for 3 weeks, replacing the medium every 3-4 days. After fixation with 4% buffered formaldehyde overnight, the pellets were embedded in 2% agarose and further processed for routine paraffin sectioning. Chondrogenic differentiation was evaluated by the Alcian blue histological staining on 8 µm thick sections.

c. *Adipogenic differentiation*

Approximately 2.1×10^4 cells/cm^2 were seeded in six-well culture dishes and cultured until 100% confluency. Cells were then exposed to four cycles of 72h culturing in the adipogenic induction medium and 24h of culturing in the adipogenic maintenance medium, followed by five consecutive days of culturing in adipogenic maintenance medium (Koch et al., 2007). After 21 days, adipogenic differentiation was assessed using Oil Red O histological staining after fixation in 4% buffered formaldehyde.

3.3.7. Flow cytometry

After Percoll isolation, MNC were cultured and subsequently, the obtained MSC were expanded for the next three passages and used at a concentration of approximately 2×10^5 cells per tube. In general, cells were washed in DMEM + 1% bovine serum albumin (BSA) and for intracellular antigen detection (CD79α and macrophage/monocyte marker), first fixed and permeabilized using Fix and Perm® (Caltag, Invitrogen) according to the manufacturer's

instructions. Cells were then incubated for 15 min at 4°C in the dark with combinations of either unlabeled or directly fluorescent-labeled monoclonal antibodies (mAbs) to obtain a multicolor analysis of the markers. The mAbs used in this study were directed against human CD29 (BioLegend, 309016), human CD44 (BioLegend, 559250), canine CD90 (VMRD, Inc., DH24A), equine MHC II (MCA1085), human CD79α (MCA2538A6) and a human macrophage/monocyte marker (MCA874A48) (all from Serotec). As positive controls, freshly isolated equine peripheral MNC and endothelial cells were used to test for species cross-reactivity and as negative controls, cells were incubated either with secondary Ab alone or with isotype controls: rat IgG2 (BioLegend, 400611) for CD29, mouse IgG1 (BioLegend, 400132) for CD44 and mouse IgM (Becton Dickinson, 557275) for CD90, respectively. After two washing steps, cells which were incubated with non-labeled mAbs were subsequently incubated with a secondary Ab for 15 min at 4°C in the dark. Secondary Abs used were R-Phycoerythrin-conjugated sheep anti-mouse IgG (Sigma, P8547) and Alexa 647-conjugated goat anti-mouse IgG (Invitrogen, A21235). Cell pellets were finally washed twice to remove the excess of secondary Ab and resuspended in 400µl phosphate buffered saline (PBS). At least 10,000 cells were analyzed using the FACScanto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser, and FACSDiva software. All data were corrected for autofluorescence using autofluorescent tubes, as well as for unspecific bindings using secondary Ab negative controls and isotype controls.

3.3.8. Statistical analysis

Data were presented as median ± interquartile range. Kruskal-Wallis tests were performed to evaluate whether the isolation method used was associated with concentration and viability. Cox proportional hazard survival models were fit to study the association between growth of

cells (1 = growth, 0 = censored) and different predictor variables (cell isolation method, cell concentration, culture medium and serum coating). Mare was forced into the model to correct for clustering. A multivariable model was built omitting non-significant (P <0.05) variables using a backward stepwise approach. Hazard ratios (HR) with 95% confidence intervals were calculated. A Kaplan-Meier graph was generated. All analyses were performed using SPSS 16.0 (SPSS Inc. Headquarters, Chicago, Illinois, US).

3.4. Results

3.4.1. Isolation of putative MSC using Percoll gradient centrifugation results in the highest number of adherent spindle-shaped cell colonies

Eight UCB samples were collected without any complication and blood cells from each sample were isolated in parallel. The cell viability was comparable for the four isolation methods (P=0.15) (Table 1). Generally, adherent spindle-shaped cell colonies occurred within 10.1 ± 4.4 days (ranging from 6 to 32 days), and 80 to 100% cell confluency was reached after 15.6 ± 1.8 days of culture. Interestingly, colony formation of UCB-derived blood cells was significantly influenced by the mare (e.g. age, parity, genetic background,…) on the one hand (P<0.001) and the isolation method used on the other hand (P<0.001) (Table 2). For the latter it was found that adherent spindle-shaped cell colonies were formed in 37 out of 96 units (38.5%) by means of the Percoll method, whereas this was only in 15 out of 96 units (15.6%) when using Ficoll-Paque (HR=0.262) (Table 2). For both the NH_4Cl and HES method, adherent colonies were observed in only 1 out of 96 (1.0%) units (HR= 0.013 and =0.017 respectively) (Table 2).

Table 1. Viability (%) and concentration of isolated umbilical cord blood cells (× 10^6 cells/mL UCB) with four separation procedures, expressed as median ± interquartile range (n=8).

	NH_4Cl	Percoll	Ficoll-Paque	HES
Viability	98.8 (98.2 - 99.4)	98.2 (97.3 - 99.1)	98.1 (96.0 - 98.8)	99.2 (99.1 - 99.6)
Concentration	42.5 (38.5 - 55.3)	1.8 (0.6 - 1.9)	2.2 (1.5 - 2.6)	22.4 (18.1 - 28.4)

The first colonies were recovered as early as six days after the start of culturing Percoll-isolated MNC and from ten days onwards, larger adherent spindle-shaped cell colonies were formed as shown by the clear increase in the percentage of units showing growth (Fig. 2). A similar pattern was also observed when culturing Ficoll-isolated MNC, but to a much lower extent (Fig. 2). Almost no growing spindle-shaped cell colonies were observed with blood cells isolated by means of either NH_4Cl or HES (Fig. 2).

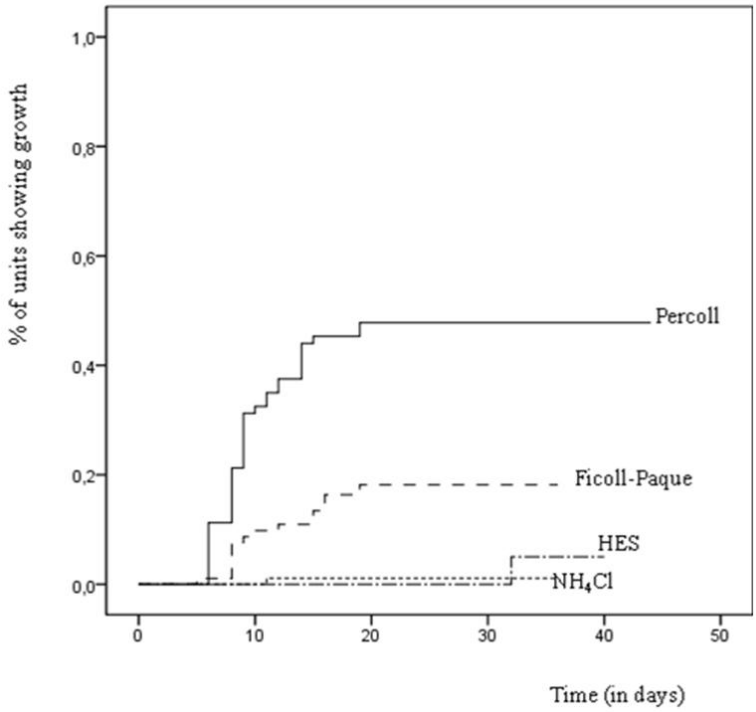

Figure 2. Growth of putative mesenchymal stromal cell units. Kaplan-Meier graph showing growth over time (days) in function of the four isolation methods used.

In contrast to the significant influence of the isolation method used, colony formation was not influenced by the original seeding concentration, nor by the absence or presence of coating, nor by the culture medium used (Table 2). When blood cells were cultured at a concentration of 4×10^6 cells/mL, colonies were noted in 19 out of 128 units (14.8%), which was not significantly different from the results obtained with the concentrations of 2×10^6 cells/mL and 1×10^6 cells/mL (16 and 19 out of 128 units (12.5% and 14.8%), respectively) (Table 2). Culturing isolated blood cells in commercial MesenCult® medium or Koch's medium resulted in similar numbers of units showing growth, namely 27 out of 192 units (14.1%) for both culture media (Table 2). Finally, seeding blood cells in FCS-coated wells resulted in growing colonies in 29 out of 192 units (15.1%), which was not significantly different from the growth rates obtained when cells were seeded in uncoated wells (25 out of 192 units (13%) (Table 2).

Table 2. Final survival model describing the variables associated with growth of MSC.

Variable	***P* value**	**HR[a]**	**95% CI[b]**	
Mare[c]	<0.001			
Method	<0.001			
Percoll[d]		1	-	-
NH_4Cl		0.013	0.002	0.095
Ficoll		0.262	0.138	0.496
HES		0.017	0.002	0.125
Density	NS[e]			
$4x10^6$ [d]		-	-	-
$2x10^6$		-	-	-
$1x10^6$		-	-	-
Medium	NS[e]			
MesenCult		-	-	-
Koch		-	-	-
Coating	NS[e]			
coated		-	-	-
uncoated		-	-	-

[a]Hazard ratio; [b]95% confidence interval around HR; [c]Data for individual mares are not shown in this table; [d]Reference category; [e]NS= non significant

3.4.2. UCB-derived putative MSC are capable of differentiation

Putative MSC were isolated in 13 out of 17 UCB samples with the optimized isolation and culture protocol as described above. In these samples, adherent spindle-shaped cell colonies started to grow within 9.9 ± 2.2 days (ranging from 7 to 14 days) and 80 % cell confluency was reached after 15.5 ± 2.2 days of culture.

From five UCB samples, putative MSC at their third passage were used to initiate *in vitro* osteogenic, chondrogenic and adipogenic differentiation. It was found that the cells were able to differentiate into osteocytes, as demonstrated by an increased alkaline phosphatase activity compared to the negative control group (Fig. 3A & D). In addition, phosphate and calcium deposits, which are osteogenic specific features, were demonstrated using Alizarine Red S and Von Kossa staining, respectively (Fig. 3B, C, E & F). Differentiation towards the chondrogenic lineage was confirmed by a positive Alcian blue staining (Fig. 4A, B & C), which identifies the acid mucins in the chondrogenic matrix. Differentiation of the MSC towards adipocytes was confirmed by a positive Oil Red O staining, which is used to detect the intracellular accumulation of lipid droplets (Fig. 5A & B).

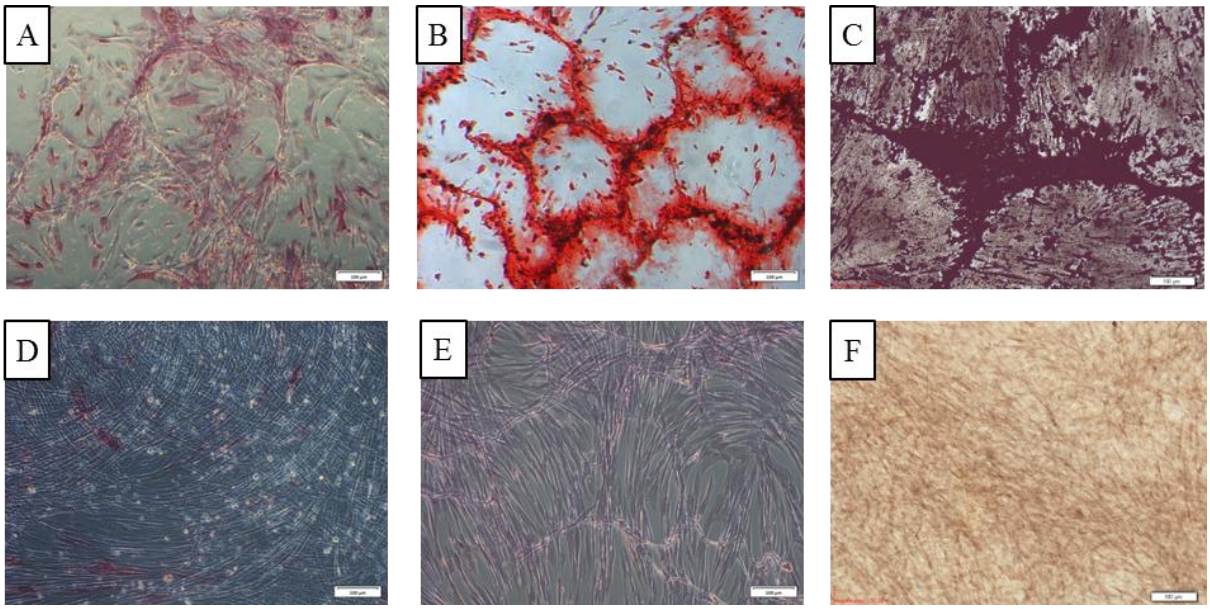

Figure 3. MSC can differentiate into osteocytes. Differentiation towards the osteogenic lineage was confirmed by increased alkaline phosphatase activity (A, 10x), Alizarine Red S (B, 10x) and Von Kossa (C, 10x) histological staining. Undifferentiated MSC from the negative control group showed a weak positivity for alkaline phosphatase activity (D, 10x) and were negative for the Alizarine Red S (E, 10x) and Von Kossa (F, 10x) staining.

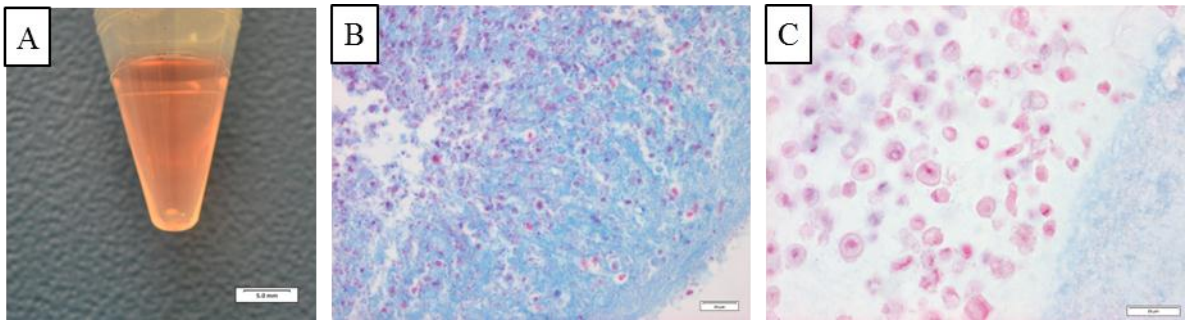

Figure 4. MSC can differentiate into chondrocytes. Differentiation towards the chondrogenic lineage was performed using a micromass culture system (A). The differentation was confirmed by Alcian Blue histological staining (B, 40x). Putative MSC which were considered as not been differentiated into chondrocytes, are shown as negative control (C, 40x).

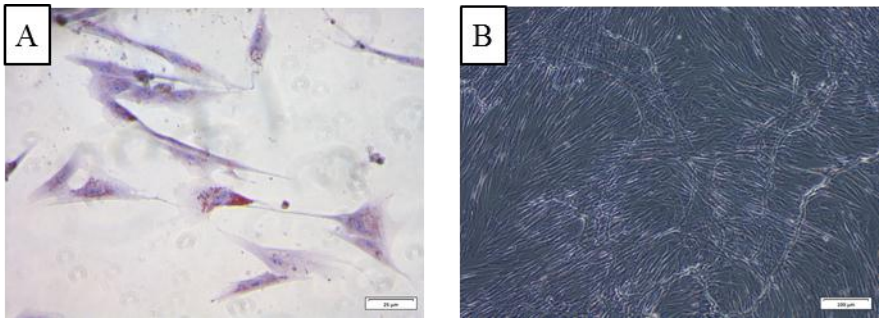

Figure 5. MSC can differentiate into adipocytes. Differentiation towards the adipogenic lineage was confirmed by Oil Red O (A, 40x) histological staining. Undifferentiated MSC from the negative control group were negative for the Oil Red O staining (B, 10x).

3.4.3. UCB-derived putative MSC exhibit required immunophenotypical characteristics

An important feature to identify solid MSC is their immunophenotyping based on the expression of a set of cell markers such as CD29, CD44, CD73, CD90 and CD105, and the absence of other cell markers which are mainly present on leukocytes and hematopoietic stem cells i.e. CD34, CD45, CD11b or CD14, CD19 or CD79α and MHC II (Dominici et al., 2006; De Schauwer et al., 2011). To this end, putative MSC from the 4th passage were used and incubated with a mixture of mAbs against human CD29, human CD44, canine CD90, equine MHC II, human CD79α and a human macrophage/monocyte marker. Hereby, it was found that the equine MSC expressed CD29, CD44 and CD90 (>90%) and lacked expression of CD79α, the macrophage/monocyte marker and MHC II (<5%) (Fig. 6). Expression of CD34, CD45, CD73 and CD105 could not be evaluated since no cross-reactivity could be demonstrated for these mAbs using the proper equine control cells (data not shown). Isolated MSC were cryopreserved, thawed and cultured for one passage, whereafter they were characterized again flow cytometrically. No significant differences were observed between these cryopreserved and fresh MSC (data not shown).

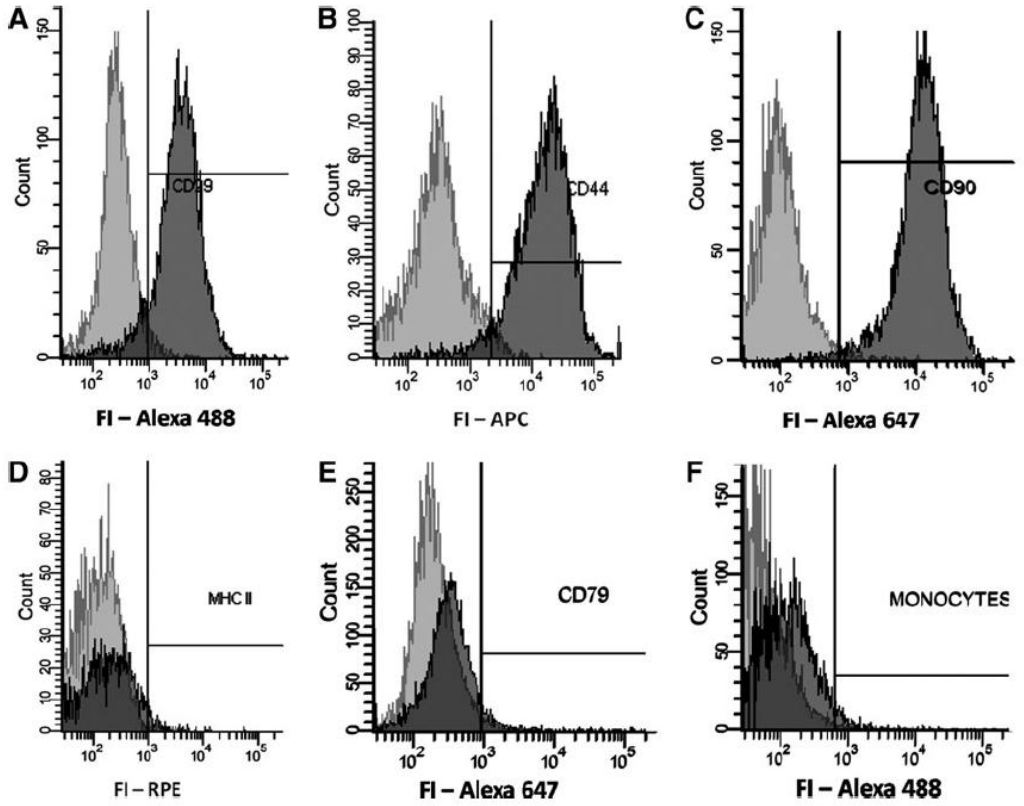

Figure 6. Immunophenotype of equine MSC. Isolated MSC were positive for CD29 (A), CD44 (B), CD90 (C) and were negative for MHC II (D), CD79α (E) and the macrophage/monocyte marker (F), as assessed by multicolor flow cytometry. A representative horse UCB sample is shown. Histograms represent relative numbers of cells vs. fluorescence intensity (FI). The light and dark grey histograms represent negative controls (autofluorescence) and test samples incubated with the mAbs, respectively.

3.5. Discussion

Isolation of human MSC from UCB has been described with varying success (Goodwin et al., 2001; Mareschi et al., 2001; Wexler et al., 2003; Bieback et al., 2004; Lee et al., 2004; Kern et al., 2006; Weiss & Troyer, 2006). The isolation step could be the main cause for this variation in outcome, as this factor has been reported to be critical when obtaining equine MSC from BM (Bourzac et al., 2010). Therefore, the major aim of the present study was to evaluate different isolation procedures to obtain putative MSC from equine UCB. Each UCB sample was subjected to four different isolation methods which is a more favorable experimental set-up than the random allocation of samples to one of the different procedures

because the influence of variation between UCB collections is avoided. Two methods of density MNC fractionation, namely Percoll and Ficoll-Paque, were included as these techniques are regularly used to obtain human MSC (Newton et al., 1993; Campos et al., 1995; Colter et al., 2000; Barry & Murphy, 2004; Bieback et al., 2004, Lee et al., 2004; Kern et al., 2006; Meyer et al., 2006). In addition, we included two techniques to obtain the whole white blood cell fraction instead of only the MNC to start the MSC culture. Red blood cells were chemically lysed using NH_4Cl which has been described as a reliable and effective alternative to density-gradient centrifugation (Horn et al., 2008). On the other hand, rouleaux formation of RBC induced by HES, first described by Rubinstein et al. (1995) for human UCB, was also included.

The proliferation of MSC varied significantly between mares which can be partly explained by differences in storage time and UCB volume as has been suggested in other studies (Ademokum et al., 1997; M-Reboredo et al., 2000). Putative equine MSC could be obtained when the MNC fraction was isolated using both density gradient separation methods. On the other hand, adherent cell colonies were only observed on a single occasion when HES and NH_4Cl were used which is in contrast to the report that used NH_4Cl for the isolation of human MSC from BM (Horn et al., 2008). Possible explanations for our lack of success to culture putative MSC after HES or NH_4Cl isolation could be the presence of polymorphonuclear cells such as neutrophils and the contamination with remaining RBC, since these factors have been reported to decrease cell adherence and proliferation (Almici et al., 1995; Schubert et al., 2009).

When comparing both density gradient isolation methods, a significantly higher number of adherent colonies was observed with Percoll in comparison to Ficoll-Paque. In addition, the percentage of colonies which subsequently proliferated was much higher. Interestingly, Percoll is not commonly used to isolate MSC from equine UCB (Hegewald et al., 2004;

Wilke et al., 2007; Colleoni et al., 2009; Bourzac et al, 2010). Why the frequently used Ficoll-Paque based isolation technique (Smith et al., 2003; Koerner et al., 2006; Vidal et al., 2006; Koch et al., 2007; Giovannini et al., 2008; Shuh et al., 2009; Martinello et al., 2010; Carrade et al., 2011) was not as successful in recovering putative equine MSC in the present study, might be due to different reasons. First, in the Percoll isolation protocol used, the UCB was first centrifuged to obtain the buffy coat fraction in contrast to the Ficoll-Paque isolation, where whole blood was used. In humans, the buffy coat fraction has been described to contain almost 70-90% of human stem cells (Seghatchian, 1999). Furthermore, the difference in chemical composition of the two gradient media may contribute to the variation in outcome of the isolation of equine putative MSC from UCB. Ficoll-Paque is based on a mixture of a synthetic sucrose polymer and an iodinated compound, whereas Percoll consists of colloidal particles coated with polyvinylpyrrolidone (Pertoft, 2000). The main disadvantages of sucrose solutions, such as Ficoll, are their physico-chemical properties including a high osmolality and viscosity (Pretlow & Pretlow, 1989) although the iodinated compound in the Ficoll-Paque used in the present study should have eliminated the high osmotic stress (Pertoft, 2000). Still, Percoll is less viscous implying a reduced risk of cell agglutination (Freshney, 2000). Furthermore, Percoll does not alter the density of monocytes which results in a better separation of lymphocytes and monocytes (Ulmer et al., 1984; Freshney, 2000).

Aside from the isolation methods, the influence of some culture parameters such as seeding density, culture media and coating of the wells with FCS was evaluated. Reported seeding densities of blood cells for MSC isolation range broadly from 1×10^4 cells/cm^2 up to 1×10^6 cells/cm^2 (Erices et al., 2000; Barry & Murphy, 2004; Bieback et al., 2004; Yen et al., 2008). A higher seeding density will lead to a higher secretion of biologically active factors by the plated cells which may contribute to cell survival as well as angiogenesis (Tang et al., 2005; Potapova et al., 2007). On the other hand, the seeding density cannot be too high either,

because non-adherent cells as well as non-cellular debris can possibly block an effective attachment of the target cells to the plastic. No significant differences were found in the recovery of putative equine MSC in the present study, indicating that a seeding concentration from 1 up to $4x10^6$ cells/mL is a good range for a proper equine MSC isolation from UCB. In addition, we did not find significant differences in putative equine MSC recovery between (i) the seeding of cells on uncoated versus FCS-coated wells and (ii) the two culture media tested. Coating with FCS was evaluated in our study because it has been used in human MSC cultures to prevent stable adherence of monocytic cells (Bieback et al., 2004). Indeed, Bieback et al. (2004) observed a significantly higher percentage of monocytes in the non-adherent fraction after plating the MNC fraction on FCS-coated wells, which indicates that fewer monocytes adhere to coated plates in comparison to uncoated plates. In contrast to the latter paper but in accordance with our findings, Koerner et al. (2006) reports variable results when wells were pre-coated with FCS. As there is the factor of variation between different batches of FCS, we decided to use non-coated wells for culturing equine MSC in the optimized protocol. Finally, two different media i.e. MesenCult® and Koch's medium, were tested because previous research indicated that human BM-derived MSC could be cultured using MesenCult®, whereas attempts to isolate human MSC from UCB using this medium failed (Bieback et al., 2004). In the present study, culturing putative MSC was successful using either medium, indicating that the medium is not a determining factor for the isolation of putative equine MSC from UCB.

Aside from the differentiation experiments, the UCB-derived equine MSC were also immunophenotypically characterized. We found that the isolated cells expressed CD29, CD44 and CD90, and were negative for CD79α, MHC II and a macrophage/monocyte marker. In general, the phenotypical identification of equine MSC is hampered by the limited availability of species-specific or cross-reacting mAbs. In the present study, a macrophage/monocyte

marker was used which detects calprotectin, an intracellular protein with a restricted distribution within the monocyte-derived cell lineage (Brandtzaeg et al., 1988). This mAb was chosen instead of mAbs recognizing CD11b or CD14, because the cross-reactivity of this mAb with equine calprotectin had already been confirmed (Perez et al., 1999), in contrast to the cross-reactivity potential of mAbs recognizing human CD11b or CD14 at the time of these experiments.

In conclusion, we describe in the present study an optimized protocol for isolating and culturing equine MSC from UCB. Importantly, the isolated equine MSC were in addition adequately characterized by assessing their differentiation capacities and immunophenotypic features. Studies comparing different isolation methods in veterinary relevant species such as the horse are of added value to the field, since Ficoll-Paque is the most commonly used density medium to isolate MSC while this present study clearly shows that Percoll gives a significantly better yield. Equine UCB can be considered to be a good and reliable source for solid equine MSC which could have a major importance in the growing field of veterinary cell-based therapies.

3.6. Acknowledgements

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References

Ademokum JA, Chapman C, Dunn J, Lander D, Mair K, Proctor SJ, Dickinson AM. Umbilical cord blood collection and separation for haematopoietic progenitor cell banking. Bone Marrow Transplant 1997;19:1023-1028.

Almici C, Carlo-Stella C, Mangoni L, Garau D, Cottafavi L, Ventura A, Armanetti M, Wagner JE, Rizzoli V. Density separation of umbilical cord blood and recovery of hematopoietic progenitor cells: implications for cord blood banking. Stem Cells 1995;13:533-540.

Almici C, Carlo-Stella C, Wagner JE, Rizzoli V. Density separation and cryopreservation of umbilical cord blood cells: evaluation of recovery in short- and long-term cultures. Acta Haematol 1996;95:171-175.

Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004;36:568-584.

Bertolini F, Lazzari L, Lauri E, Corsini C, Castelli C, Gorini F, Sirchia G. Comparative study of different procedures for the collection and banking of umbilical cord blood. J Hematother 1995;4: 29-36.

Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004;22:625-634.

Bourzac C, Smith LC, Vincent P, Beauchamp G, Lavoie J-P, Laverty S. Isolation of equine bone marrow-derived mesenchymal stem cells: a comparison between three protocols. Equine Vet J 2010;42:519-527.

Brandtzaeg P, Jones DB, Flavell DJ, Fagerhol MK. Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. J Clin Pathol 1988;41:963-970.

Campos L, Roubi N, Guyotat D. Definition of optimal conditions for collection and cryopreservation of umbilical cord hematopoietic cells. Cryobiology 1995;32:511-515.

Carrade DD, Owens SD, Galuppo LD, Vidal MA, Ferraro GL, Librach F, Buerchler S, Friedman MS, Walker NJ, Borjesson DL. Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses. Cytotherapy 2011;13:419-430.

Colleoni S, Bottani E, Tessaro I, Mari G, Merlo B, Romagnoli N, Spadari A, Galli C, Lazzari G. Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor. Vet Res Commun 2009;33,811-821.

Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc. Natl. Acad. Sci 2000;97:3213-3218.

Covas DT, Siufi JLC, Silva ARL, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. Braz J Med Biol Res 2003;36:1179-1183.

De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011;75:1431-1443.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.

Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000;109:235-242.

Freshney RI. Cell separation. In: Freshney, R.I. Culture of Animal Cells: a manual of basic technique. New York, Wiley-Liss, 2000, pp. 215-229.

Giovannini S, Brehm W, Mainil-Varlet P, Nesic D. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. Differentiation 2008;76:118-129.

Goodwin HS, Bicknese AR, Chien S-N, Bogucki BD, Oliver DA, Quinn CO, Wall DA. Multilineage differentiation activity by cells isolated from umbilical cord blood: expression of bone, fat, and neural markers. Biol Blood Marrow Transplant 2001;7:581-588.

Harris DT, Schumacher MJ, Rychlik S, Booth A, Acevedo A, Rubinstein P, Bard J, Boyse EA. Collection, separation and cryopreservation of umbilical cord blood for use in transplantation. Bone Marrow Transplant 1994;13:135-143.

Hegewald AA, Ringe J, Bartel J, Krüger I, Notter M, Barnewitz D, Kaps C, Sittinger M. Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study. Tissue Cell 2004;36:431-438.

Horn P, Bork S, Diehlmann A, Walenda T, Eckstein V, Ho AD, Wagner W. Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis. Cytotherapy 2008;10:676-685.

Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T, Mitchell KE. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem Biophys Res Commun 2007;362:347-353.

Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006;24:1294-1301.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

Koch TG, Thomsen PD, Betts DH. Improved isolation protocol for equine cord blood-derived mesenchymal stromal cells. Cytotherapy 2009;11:443-447.

Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP. Equine peripheral blood -derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. Stem Cells 2006;24:1613-1619.

Lee OK, Kuo TK, Chen W, Lee K, Hsieh S, Chen T. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004;103:1669-1675.

Leppänen M, Tulamo R-M, Heikkilä P, Katiskalahti T. Follow-up of equine tendon and ligament injuries 18-24 months after treatment with enriched autologous adipose-derived mesenchymal stem cells – a clinical study. Regen Med 2009;4(6):S21-S22.

Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F. Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica 2001;86:1099-1100.

Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M. Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood. Tissue Eng Part C 2010;16:771-781.

Meyer TPH, Hofmann B, Zaisserer J, Jacobs VR, Fuchs B, Rapp S, Weinauer F, Burkhart J. Analysis and cryopreservation of hematopoietic stem and progenitor cells from umbilical cord blood. Cytotherapy 2006;8:265-276.

M-Reboredo N, Diaz A, Castro A, Villaescusa RG. Collection, processing and cryopreservation of umbilical cord blood for unrelated transplantation. Bone Marrow Transplant 2000;26:1263-1270.

Newton I, Charbord P, Schaal JP, Herve P. Toward cord blood banking: density-separation and cryopreservation of cord blood progenitors. Exp Hematol 1993;21:671-674.

Panepucci RA, Siufi JLC, Silva Jr WA, Proto-Siquiera R, Neder L, Orellana M, Rocha V, Covas DT, Zago MA. Comparison of gene expression of umbilical cord vein and bone marrow- derived mesenchymal stem cells. Stem Cells 2004;22:1263-1278.

Perez J, Mozos E, Martin MP, Day MJ. Immunohistochemical study of the inflammatory infiltrate associated with equine squamous cell carcinoma. J Comp Path 1999;121:385-397.

Pertoft H. Fractionation of cells and subcellular particles with Percoll. J Biochem Biophys Methods 2000;44:1-30.

Potapova IA, Gaudette GR, Brink PR, Robinson RB, Rosen MR, Cohen IS, Doronin SV. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells *in vitro*. Stem Cells 2007;25:1761-1768.

Pretlow TG, Pretlow TP. Cell separation by gradient centrifugation methods. Methods Enzymol 1989;171:462-482.

Quillen K, Berkman EM. Methods of isolation and cryopreservation of stem cells from cord blood. J Hematother 1996;5:153-155.

Regidor C, Posada M, Monteagudo D, Garaulet C, Somolinos N, Forés R, Briz M, Fernandez MN. Umbilical cord blood banking for unrelated transplantation: evaluation of cell separation and storage methods. Exp Hematol 1999;27:380-385.

Richardson LE, Dudhia J, Clegg PD, Smith R. Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury. Trends Biotechnol 2007;25:409-416.

Rubinstein P, Dobrila L, Rosenfield RE, Adamson JW, Migliaccio G, Miggliaccio AR, Taylor PE, Stevens CE. Processing and cryopreservation of placental/umbilical cord blood for unrelated bone marrow reconstitution. Proc. Natl. Acad. Sci 1995;92:10119-10122.

Schubert R, Geiger H, Zielen S, Baer PC. Simultaneous detection of ERK-, p38-, and JNK-MAPK phosphorylation in human adipose-derived stem cells using the cytometric bead array technology. J Immunol Meth 2009;350:200-204.

Seghatchian MJ. What's happening? Cord blood derived stem cells: current views. Transfus Sci 1999;20:141-142.

Shuh EM, Friedman MS, Carrade DD, Li J, Heeke D, Oyserman SM, Galuppo LD, Lara DJ, Walker NJ, Ferraro GL, Owens SD, Borjesson DL. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical-cord blood. Am J Vet Res 2009;70:1526-1535.

Smith RKW, Korda M, Blunn GW, Goodship AE. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. Equine Vet J 2003;35:99-102.

Strober W. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 2001 Appendix 3, Appendix 3B.

Tang YL, Zhao Q, Qin X, Shen L, Cheng L, Ge J, Philips MI. Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. Ann Thorac Surg 2005;80:229-237.

Ulmer AJ, Scholz W, Ernst M, Brandt E, Flad HD. Isolation and subfractionation of human peripheral blood mononuclear cells by density gradient centrifugation on Percoll. Immunobiology 1984;3:238-250.

Vidal MA, Kilroy GE, Johnson JR, Lopez MJ, Moore RM, Gimble JM. Cell growth characteristics and differentiation frequency of adherent equine bone marrow-derived mesenchymal stromal cells: adipogenic and osteogenic capacity. Vet Surg 2006;35:601-610.

Weiss ML, Troyer DL. Stem cells in the umbilical cord. Stem Cell Rev. 2006;2:155-162.

Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol 2003;121:368-374.

Wilke MM, Nydam DV, Nixon AJ. Enhanced early chondrogenesis in articular defects following arthroscopic mesenchymal stem cell implantation in an equine model. J Orthop Res 2007;25(7):913-925.

Yen BL, Chien C, Chen Y, Chen J, Huang J, Lee FK, Huang H.I. Placenta-derived multipotent cells differentiate into neuronal and glial cells *in vitro*. Tissue Eng Part A 2008;14:9-17.

CHAPTER 4

OPTIMIZATION OF IMMUNOPHENOTYPING EQUINE MESENCHYMAL STROMAL CELLS ISOLATED FROM UMBILICAL CORD BLOOD

De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JLJ, Braeckmans K, Van Soom A, Meyer E. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry part A 2012;81(4):312-23.

4.1. Abstract

During recent years, cell-based therapies using mesenchymal stromal cells (MSC) are reported in equine veterinary medicine with increasing frequency. In most cases, the isolation and *in vitro* differentiation of equine MSC is described but their proper immunophenotypic characterization is rarely performed. The lack of a single marker specific for MSC and the limited availability of monoclonal antibodies (mAbs) for equine MSC in particular, strongly hamper this research. In the present study, 30 commercial mAbs were screened with flow cytometry for recognizing equine epitopes using the appropriate positive controls to confirm their specificity. Cross-reactivity was found and confirmed by confocal microscopy for CD45, CD73, CD79α, CD90, CD105, MHC II, a monocyte marker and two clones tested for CD29 and CD44. Unfortunately, none of the evaluated CD34 clones recognized the equine epitopes on positive control endothelial cells. Subsequently, umbilical cord blood (UCB)-derived undifferentiated equine MSC of the fourth passage of six horses were characterized using multicolor flow cytometry based on the selected 9-marker panel of both cell surface antigens and intracytoplasmatic proteins. In addition, appropriate positive and negative controls were included and the viable single cell population was analyzed by excluding dead cells using 7-AAD. Isolated equine MSC of the fourth passage were found to be CD29, CD44, CD90 positive and CD45, CD79α, MHC II and a monocyte marker negative. A variable expression was found for CD73 and CD105. Successful differentiation towards the osteogenic, chondrogenic and adipogenic lineage was used as additional validation. We suggest that this selected 9-marker panel can be used for the adequate immunophenotyping of equine MSC.

4.2. Introduction

The considerable therapeutic potential of equine MSC in regenerative medicine has generated a markedly increasing interest in this research area (Koch et al., 2007; Reed & Johnson, 2008). In equine veterinary medicine, MSC are used experimentally for the treatment of tendon, ligament, and cartilage injuries (Fortier & Travis, 2011). Currently, no medical treatments are available to reverse cartilage injuries (Koch et al., 2007). For tendon injuries, the scar tissue formed during the repair is functionally deficient, which has tremendous consequences for the horse in terms of reduced performance and a considerable risk for re-injury (Richardson et al., 2007). For example, Pacini et al. (2007) demonstrated in a case control study that 9 out of 11 Italian racehorses treated with MSC derived from bone marrow, successfully returned to their athletic level before injury. Moreover, during a two year follow-up period, no re-injury of the superficial digital flexor occurred in the treated group in contrast with the control group horses which were all re-injured (Pacini et al., 2007).

However, before any type of stem cell can be applied in practice, its unequivocal characterization by a set of specific functional or phenotypic markers is crucial (Tarnok et al., 2010). In contrast to the criteria of the International Society for Cellular Therapy (ISCT) which were defined to identify human MSC (Dominici et al., 2006), there is a lack of uniformity to characterize equine MSC in veterinary medicine (De Schauwer et al., 2011a). For human MSC, it has been defined that these cells must be plastic-adherent and be capable of differentiating towards the osteogenic, chondrogenic and adipogenic lineage. Furthermore, they must express CD29, CD44, CD73, CD90, and CD105 and lack expression of CD14, CD34, CD45, CD79α and MHC II. Although there have been several reports on the isolation and *in vitro* differentiation of equine MSC, few research groups have attempted to identify a set of immunophenotypic markers to characterize these cells (Hoynowski et al., 2007; Guest et al., 2008; Braun et al., 2010; Martinello et al., 2010; Radcliffe et al., 2010). The lack of a

single marker specific for MSC and the currently limited availability of monoclonal antibodies (mAbs) for immunophenotyping equine cells, are major factors complicating the progress of this type of research. To our knowledge, commercial mAbs which are directed against equine epitopes are only available for CD44 and MHC II (Serotec, Düsseldorf, Germany). Consequently, for other equine MSC markers, candidate non-equine mAbs should be evaluated in search for cross-reactivity.

In stem cell research in general and MSC research in specific, immunophenotyping is preferably performed by multicolor flow cytometry to simultaneously demonstrate the co-expression of specific MSC markers and the absence of hematopoietic antigen expression (Dominici et al., 2006; Xie et al., 2010). However, many flow cytometric techniques have been developed for analyzing mature, non-adherent leucocytes and therefore, some refinements are required when using stem cells (Hughes et al., 2009). For example, the use of gating strategies is not only important to select the population of interest based on the selected markers but also to ensure an accurate analysis of the obtained data by excluding aggregates. Furthermore, isotype controls and/or unstained cells are imperative to make a clear distinction between fluorescent positive and negative populations (Hughes et al., 2009).

In the current study, 30 commercially available mAbs were first validated for recognizing equine epitopes using equine mononuclear cells (MNC), equine lymphocytes or equine endothelial cells as appropriate positive control cells. If required, additional experiments on human cells as reference positive control were performed. Confocal microscopy validated the flow cytometric results for all cross-reacting mAbs. Subsequently, equine umbilical cord blood (UCB)-derived MSC of six horses were characterized by the selected panel of 9 mAbs, based on their cross-reactivity with equine epitopes as determined in the first part of the study. Cells from the fourth passage were used to perform these immunophenotyping experiments.

4.3. Materials and methods

4.3.1. Isolation of human and equine cells

Equine peripheral blood was obtained from ten horses between 8 and 12 years, all female and healthy. Human peripheral blood was obtained from three healthy male humans between 29 and 39 years old with informed consent. Equine MSC were isolated from six mares between 5 and 18 years old which had foaled in the Reproduction Clinic of the Faculty of Veterinary Medicine with informed consent of the owner. The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2010/147).

After collecting whole blood into a vacuum blood tube, human and equine MNC were isolated using a Percoll® (density 1.080 - GE Healthcare, Little Chalfont, United Kingdom) gradient while human and equine lymphocytes were isolated using Ficoll-Paque® (density 1.077 - GE Healthcare, Little Chalfont, United Kingdom), according to the manufacturer's instructions. Cells were resuspended in Dulbecco's modified eagle medium (DMEM) + 1% bovine serum albumin (BSA) (Invitrogen, Gent, Belgium). To isolate primary equine vascular endothelial cells from the *A. carotis* of healthy horses, collagenase (Type II, Sigma, Bornem, Belgium) treatment was used as described previously (MacEachern et al., 1997; Van de Walle et al., 2008). To separate the endothelial cells from smooth muscle cells and fibroblasts, cultures were labeled with 10 µg/mL low density lipoprotein 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanide perchlorate (Biomedical Technologies Inc, Stoughton, USA) for 4h at 37°C. After trypsinization, endothelial cells were washed, resuspended in media and sorted by fluorescence-activated cell sorting (Van de Walle et al., 2008).

Equine MSC derived from UCB were isolated and cultured as previously described (De Schauwer et al., 2011b). Briefly, MNC were isolated from the UCB using a Percoll® (GE Healthcare, Little Chalfont, United Kingdom) gradient and cultured at a concentration of

4×10^6 cells/mL in uncoated T-25 culture flasks. The isolated cells were incubated at 38.5°C in a humidified atmosphere containing 5% CO_2. Cells were passaged as soon as confluency exceeded 80% using 0.083% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma, Bornem, Belgium). For this purpose, the adherent MSC were washed with Hank's buffered salt solution (HBSS) without Ca/Mg (Invitrogen, Gent, Belgium) during 5 min, and subsequently incubated with trypsin-EDTA during 5 min at 37°C. Cold culture medium containing fetal calf serum (FCS) was added to block the action of the trypsin after which the cell suspension was centrifuged during 8 min at 300 ×g. Finally, the cell pellet was resuspended in culture medium and concentration and cell viability were determined using trypan blue exclusion (Strober et al., 2001).

After two passages, approximately one million undifferentiated MSC were used to differentiate towards the osteogenic, chondrogenic and adipogenic lineage and thus confirmed the MSC identity, as previously described (De Schauwer et al., 2011b). Briefly, after 20 days of culture in osteogenic medium, osteogenic differentiation was evaluated using the Alizarine Red S and the Von Kossa histological staining, as well as by detecting alkaline phosphatase activity (Millipore®, Overijse, Belgium) (Fig. 1A-C). Chondrogenic differentiation was evaluated by the Alcian blue histological staining after three weeks of culture in chondrogenic medium using a micromass culture system (Fig. 2A). Finally, the adipogenic differentiation was assessed using Oil Red O histological staining after four cycles of 72h culturing in the adipogenic induction medium and 24h of culturing in the adipogenic maintenance medium, followed by five consecutive days of culturing in adipogenic maintenance medium (Fig. 3A). For the three lineages, non-induced cells in expansion medium were used as negative controls (Fig. 1 D-F, Fig. 2B, Fig. 3B).

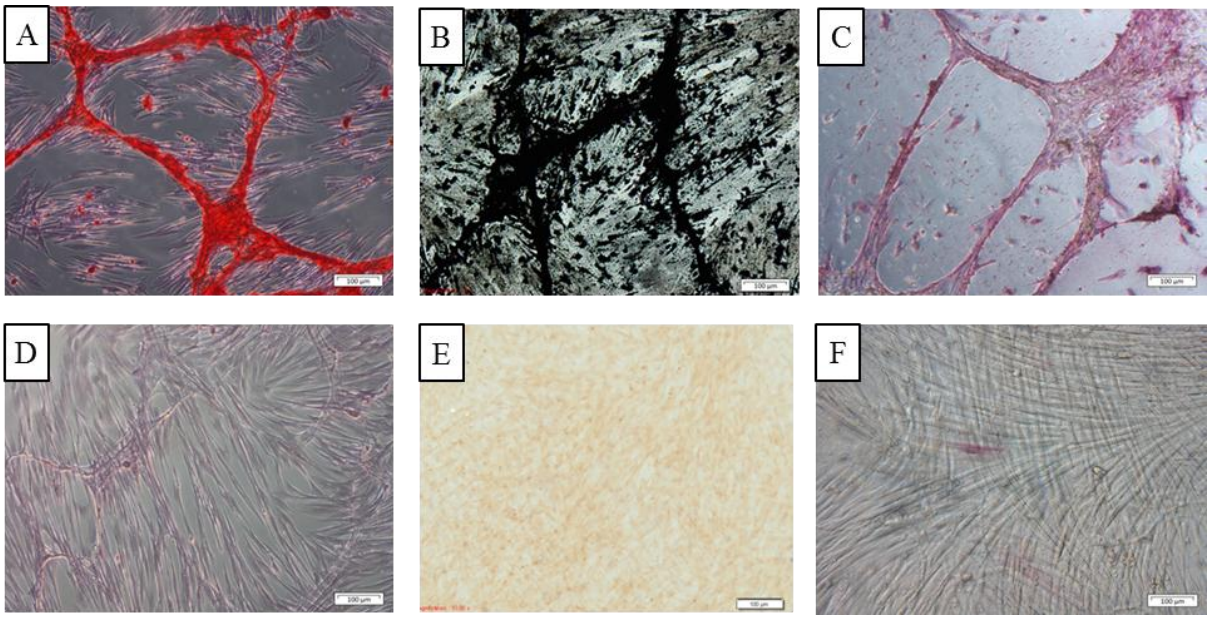


Figure 1. The osteogenic differentiation potential of the equine UCB-derived MSC as confirmed using the Alizarine Red S (A, 10x) and the Von Kossa histological staining (B, 10x), as well as by detecting alkaline phosphatase activity (C, 10x). Non-induced cells in expansion medium were used as negative controls (D-F, respectively, 10x).

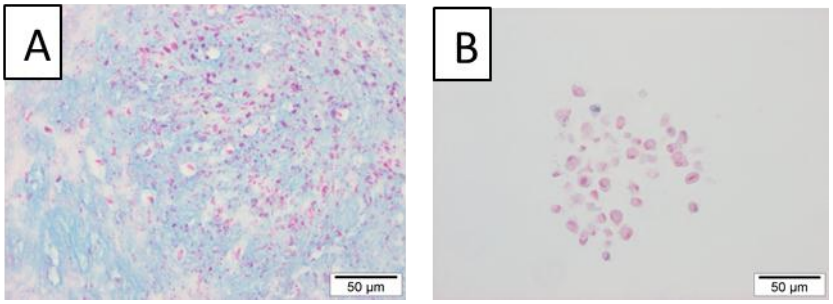


Figure 2. The chondrogenic differentiation potential of the equine UCB-derived MSC as evaluated by the Alcian blue histological staining (A). Putative MSC which were considered as not been differentiated into chondrocytes, are shown as negative control (B).

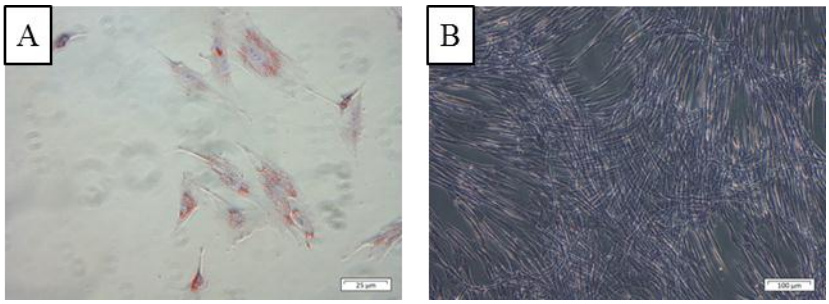


Figure 3. The adipogenic differentiation potential of the equine UCB-derived MSC as confirmed using the Oil Red O histological staining (A, 40x). Non-induced cells in expansion medium were used as negative controls (B, 10x).

4.3.2. Monoclonal antibodies

The mAbs used in this study to test for cross-reactivity were directed against CD29, CD34, CD44, CD45, CD73, CD79α, CD90, CD105, MHC II and a monocyte marker. A full list of all clones tested, the species and the companies can be found in Table 1. Secondary Abs included RPE-conjugated sheep anti-mouse IgG (Sigma, Bornem, Belgium) and Alexa 647-conjugated goat anti-mouse IgG (Invitrogen, Gent, Belgium). The isotype controls in this study included rat IgG2b, mouse IgG2a, mouse IgG1 (all from BioLegend, Uithoorn, The Netherlands) and mouse IgM (Becton Dickinson, Erembodegem, Belgium) (Table 2).

4.3.3. Single-color flow cytometry

To screen for cross-reactivity, approximately 2×10^5 cells per tube were centrifuged in DMEM + 1% BSA and incubated for 15 min at 4°C in the dark with each of the primary mAbs (Table 1). After two washing steps, cells which were incubated with non-labeled primary mAbs, were incubated with the RPE-conjugated sheep anti-mouse IgG secondary Ab for 15 min at 4°C in the dark. After three washing steps, cell pellets were finally resuspended in 400µl phosphate buffered saline (PBS) and analyzed after 10 min incubation with 7-aminoactinomycin D (7-AAD), a viability dye which is excluded by viable cells but can

Table 1. Overview of the primary monoclonal antibodies (mAbs) used in the present study and their cross-reactivity. Each mAb was tested using the appropriate equine positive control cells. All data were compensated and corrected for autofluorescence as well as for non-specific binding.

Host	Immuno-Epitope	Abs	Clone	Company	Concentration (µg/mL)	Dilutions tested	Equine positive control cells	Cross-reactivity
Mo	Hu	CD29- Alexa 488	TS2/16	Biolegend	500	1:16.6,1:25,1:50,1:100,1:200	MNC	+
Mo	Hu	CD29-PE	4B4	Beckman Coulter	ascites	1:25,1:50,1:100	MNC	+
Mo	Hu	CD34- Alexa 647	4H11	Biolegend	ascites	1:10, 1:20	EC	-
Mo	Hu	CD34-PE	581	Beckman Coulter	ascites	1:3,1:6, 1:10	EC	-
Rat	Mo	CD34-FITC	MEC14.7	Serotec	ascites	1:10	EC	-
Mo	Hu	CD34-RPE	AC136	Miltenyi	ascites	1:5,1:10	EC	-
Mo	Hu	CD34-PE	8G12	Becton Dickinson	25	1:2, 1:5	EC	-
Mo	Ho	CD44	CVS18	Serotec	ascites	1:10	MNC	+
Rat	Mo	CD44-APC	IM7	Becton Dickinson	200	1:10,1:20,1:40,1:80	MNC	+
Mo	Hu	CD45-PE	5B1	Miltenyi	ascites	1:5,1:10	MNC	-
Mo	Hu	CD45-FITC	35-Z6	Santa Cruz	200	1:3.3,1:5,1:10,1:20,1:40	MNC	-
Mo	Hu	CD45	B-A11	Abcam	ascites	1:33,1:50,1:100,1:200,1:400	MNC	-
Mo	Hu	CD45-Alexa488	F10-89-4	Serotec	ascites	1:2, 1:2.5, 1:5	MNC	+
Rat	Mo	CD45-FITC	30-F11	Becton Dickinson	ascites	1:25	MNC	-
Mo	Hu	CD45-FITC	HI30	Becton Dickinson	ascites	1:2.5, 1:5	MNC	-
Mo	Hu	CD45- APC-H7	2D1	Becton Dickinson	ascites	1:10, 1:20	MNC	-
Mo	Hu	CD73	4G4	Hycult	100	1:6.25,1:12.5,1:25,1:50	L	-
Rat	Mo	CD73-PE	496406	R&D	25	1:0, 1:2,1:4	L	-

Host	Immuno-Epitope	Abs	Clone	Company	Concentrations (µg/mL)	Dilutions tested	Equine positive control cells	Cross-reactivity
Mo	Hu	CD73-PE	AD2	Biolegend	ascites	1:5, 1:12.5, 1:25	L	-
Mo	Hu	CD73	10f1	Abcam	ascites	1:5,1:10,1:50,1:100,1:200	L	+
Mo	Hu	CD73	7G2	Abcam	500	1:25	L	-
Mo	Hu	CD79α-Alexa647	HM57	Serotec	ascites	1:2.5,1:5	MNC	+
Mo	Dog	CD90	DH24A	VMRD	ascites	1:33.3,1:66.6,1:100,1:133.3, 1:266.6	MNC	+
Mo	Hu	CD105-PE	SN6	Serotec	ascites	1:25, 1:50	EC	+
Mo	Hu	CD105-Alexa 488	43A3	Biolegend	ascites	1:10, 1:20	EC	-
Rat	Mo	CD105-Alexa488	MJ7/18	Biolegend	500	1:11.1,1:16.6,1:25,1:33.3,1:50, 1:100,1:200	EC	-
Mo	Hu	CD105	266	Becton Dickinson	500	1:16.6,1:25,1:50,1:100,1:200	EC	-
Mo	Hu	CD105	35/CD105	Becton Dickinson	250	1:8.3,1:12.5,1:25,1:50,1:100	EC	-
Mo	Ho	MHC II	CVS20	Serotec	ascites	1:50,1:100	MNC	+
Mo	Hu	Monocytesmarker-Alexa488	MAC387	Serotec	ascites	1:2.5,1:5	MNC	+

Mo: Mouse; Ho: Horse; Hu: Human; L: Lymphocytes; MNC: mononuclear cells; EC: endothelial cells

penetrate cell membranes of dying or dead cells. For intracellular antigen detection, i.e. when using the mAb directed against CD79α or the monocyte marker, cells were fixed and permeabilized first using Fix and Perm® (Caltag, Invitrogen, Gent, Belgium) according to the manufacturer's instructions. In addition, an incubation step for 15 min at room temperature (RT) in the dark with 10% horse serum was included to block non-specific binding of these mAbs to equine epitopes. No 7-AAD staining was performed on these fixed and permeabilized cells.

For all tubes, at least 10,000 cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems, Erembodegem, Belgium) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser, and FACSDiva software. All data were corrected for autofluorescence as well as for unspecific bindings using either secondary Ab and/or isotype-matched negative controls. All isotypes were matched to the immunoglobulin subtype, conjugated to the same fluorochrome and used at the same fluorescence/protein concentration as the corresponding Ab.

Table 2. Overview of the marker panels of primary mAbs and 7-AAD selected in the present study to immunophenotype viable equine UCB-derived MSC using multicolor flow cytometry. Additionally, the relevant isotype controls as well as the secondary antibodies for the indirectly labeled markers are also provided with their corresponding fluorochrome.

	subset	Marker	Clone	Sec Ab	Dilution
Multicolor FCM	1	CD29-Alexa488	TS2/16		1:20
		MHC II	CVS20	Anti-mouse RPE	1:50
		7-AAD			
		CD44-APC	IM7		1:20
	2	CD105-RPE	SN6		1:10
		7-AAD			
		CD90	DH24A	Anti-mouse Alexa647	1:100
	3	CD45-Alexa488	F10-89-4		1:5
		CD73	10f1	Anti-mouse RPE	1:5
		7-AAD			
	4	Monocyte-Alexa488	MAC387		1:2.5
		CD79α-Alexa647	HM57		1:2.5
Secondary Ab	1 & 3	Sheep anti-mouse IgG-RPE			1:20
	2	Goat anti-mouse IgG-Alexa647			1:200
Isotype controls	1&4	Mouse IgG1-Alexa488			1:20
	1&2&3	Mouse IgG1-RPE			1:10
	1	Rat IgG2b-APC			1:20
	2	Mouse IgM		Anti-mouse Alexa647	1:50
	3	Mouse IgG2a-Alexa488			1:20
	4	Mouse IgG1-Alexa647			1:100

4.3.4. Confocal immunofluorescence microscopy

The specificity of the cross-reactive mAbs was confirmed by confocal fluorescence microscopy. Briefly, the staining procedure was performed as described above for the single-color flow cytometry, with the exception of the use of the RPE-conjugated rabbit anti-mouse Ig as secondary Ab which was replaced by FITC-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark). Subsequently, cells were fixed using Celfix® (Becton Dickinson, Erembodegem, Belgium) and incubated at 4°C for 20 min in the dark. After centrifugation and resuspension in 300µl PBS, propidium iodide (PI) (10µg/mL) was added to visualize the cell nuclei and incubated for 20 min at 4°C in the dark. Following centrifugation and a

washing step, the cells were resuspended in 100µl PBS and after cytocentrifugation (Shandon, Southern Products Ltd, Runcorn, UK), the stained cells were screened using confocal fluorescence microscopy (Nikon EZ-C1, Amstelveen, the Netherlands).

4.3.5. Multicolor flow cytometry

For the multicolor flow cytometry, undifferentiated equine MSC from the fourth passage were incubated with following combinations of marker panels: CD29-Alexa488/MHC II-RPE /CD44-APC/7-AAD (subset 1), CD105-RPE/CD90-Alexa647/7-AAD (subset 2), CD45-Alexa488/CD73-RPE/7-AAD (subset 3), and the monocyte marker-Alexa488/CD79α-Alexa647 (subset 4). To identify the percentage of viable cells, 7-AAD was used in subset 1, 2 and 3, but not in subset 4 since cells in the latter subset were permeabilized. A detailed description of mAb clones and dilutions used can be found in Table 2.

In the subsets 1-3 for the cell surface markers, approximately 2×10^5 cells per tube were centrifuged to pellet in DMEM + 1% BSA and incubated for 15 min at 4°C in the dark with following non-labeled primary mAbs: MHC II (subset 1), CD90 (subset 2), and CD73 (subset 3), respectively. After two washing steps, cells which were incubated with these non-labeled primary mAbs, were incubated with a secondary Ab conjugated with a relevant fluorochrome for 15 min at 4°C in the dark (Table 2). Cell pellets were washed twice to remove the excess of secondary Ab and subsequently treated with a 15-min blocking step using 10% mouse serum to exclude non-specific binding of the directly labeled primary mAbs on the secondary Ab. Next, these directly labeled primary mAbs, i.e. CD29 and CD44 (subset 1), CD105 (subset 2), and CD45 (subset 3), respectively, were incubated for 15 min at 4°C in the dark. After three washing steps, cell pellets were finally resuspended in 400µl PBS and analyzed after 10 min incubation with 7-AAD for all three subsets of markers. For the intracellular antigen detection in subset 4, cells were first fixed and permeabilized using Fix and Perm®

(Caltag, Invitrogen, Gent, Belgium) according to the manufacturer's instructions. Subsequently, cells were preincubated with 10% horse serum during 15 min in the dark at RT as a blocking step, after which the CD79α and the monocyte marker primary mAbs were incubated for 15 min at 4°C in the dark. Finally, the pellet was resuspended in 400µl PBS after three washing steps.

For all tubes, at least 10,000 cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser, and FACSDiva software. All data were compensated and corrected for autofluorescence as well as for unspecific bindings using both secondary Ab and/or isotype negative controls. Compensation for spectral overlap between fluorochromes was performed using an automatic calibration technique (FACSDiva software, Becton Dickinson) and subsequently evaluated individually with a matrix.

4.3.6. Gating strategy

A primary gate was placed on the area versus width signal of the forward scatter (FSC-A/FSC-W) dot plot, after which this population was visualized on the area versus width signal of the side scatter (SSC-A/SSC-W) dot plot to discriminate for doublets and clumps. The single cell population was identified by defining the gated population on a side scatter area signal versus a forward scatter area (SSC-A/FSC-A) signal dot plot. The final gate for analysis was a Boolean gate on the single cell population and the 7-AAD^{neg} cells, enabling the analysis of a viable single cell population.

4.3.7. Statistical analysis

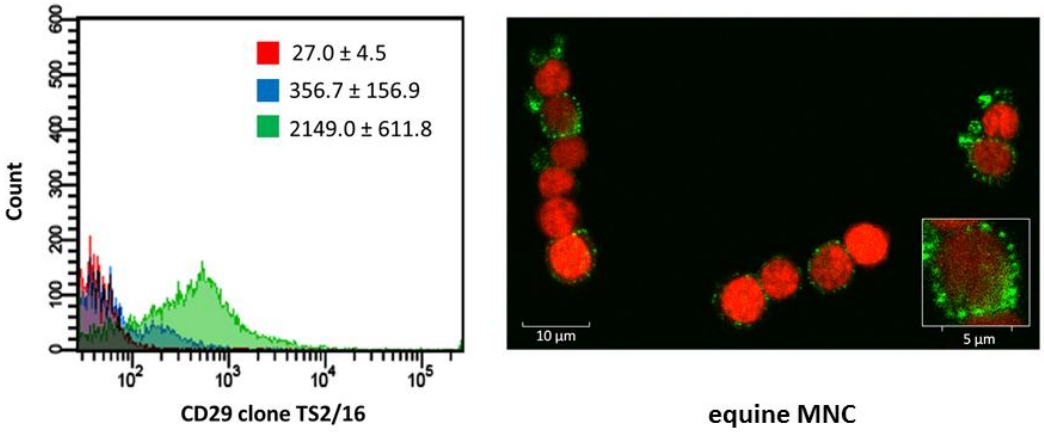
All data were analyzed using the dedicated FACSDiva software (Becton Dickinson) and subsequently exported to Excel (Excel 2007, Microsoft Corp., Redmond, WA) to calculate

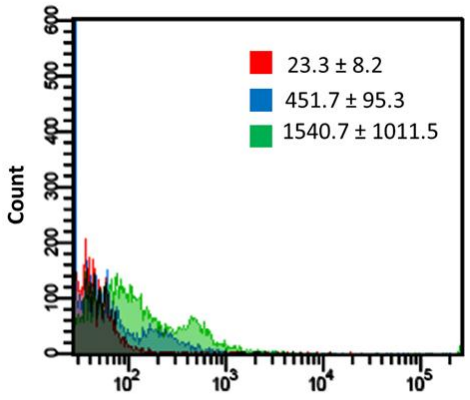
different parameters such as mean, median, standard error of the mean (SEM) and interquartile range (IQR).

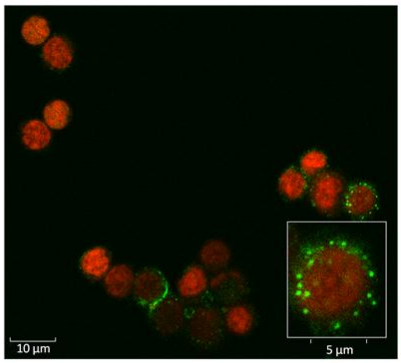
4.4. Results

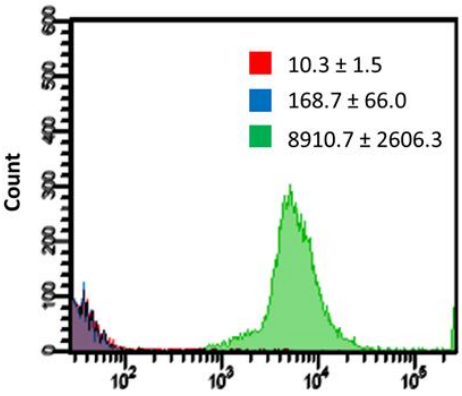
4.4.1. Assessment of antibody cross-reactivity with equine epitopes

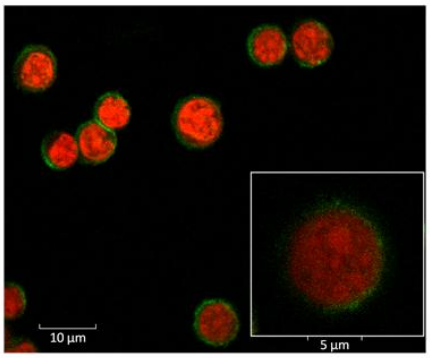
Freshly isolated equine MNC, lymphocytes and primary endothelial cells were used to validate the cross-reactivity of mAbs that are directed against human, murine and/or canine molecules, listed in Table 1. Equine MNC were used to demonstrate cross-reactivity of the mAbs directed against CD29, CD44, CD45, CD79α, CD90, MHC II and a monocyte marker, while equine lymphocytes were used to detect cross-reactivity for CD73. An increase in mean fluorescence intensity (MFI), as compared to the negative controls, indicated positivity and was detected for all screened clones directed against CD29, CD44, CD79α, CD90, MHC II and the monocyte marker (Table 1, Fig. 4). Confocal fluorescence microscopy was successfully used to validate the flow cytometric data (Fig. 4).

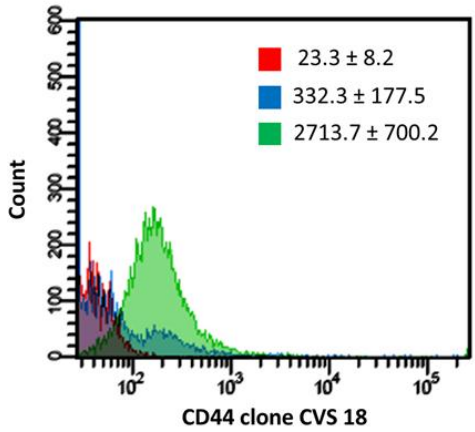


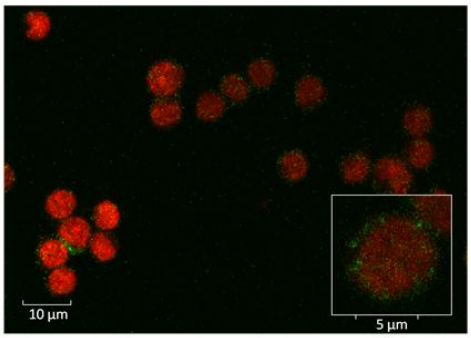

23.3 ± 8.2
451.7 ± 95.3
1540.7 ± 1011.5
Count
CD29 clone 4B4

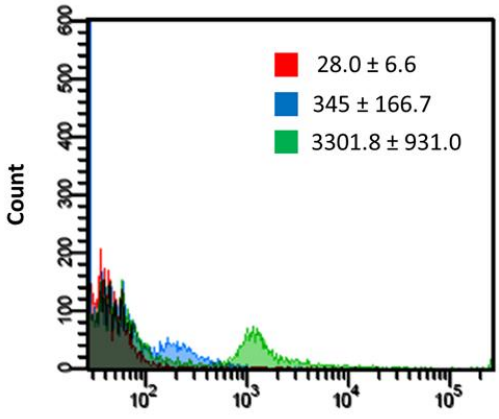

10 µm
5 µm
equine MNC

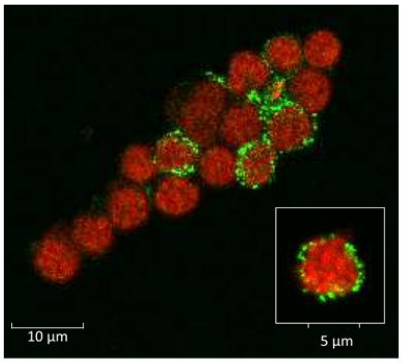

10.3 ± 1.5
168.7 ± 66.0
8910.7 ± 2606.3
Count
CD44 clone IM7

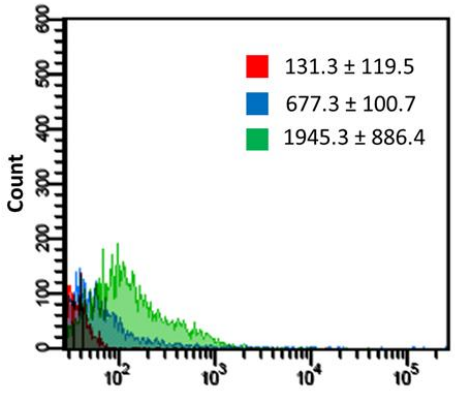

10 µm
5 µm
equine MNC

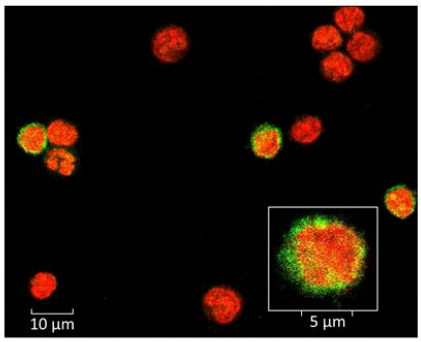

23.3 ± 8.2
332.3 ± 177.5
2713.7 ± 700.2
Count
CD44 clone CVS 18

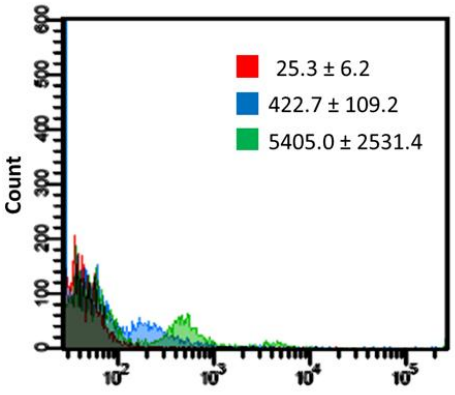

10 µm
5 µm
equine MNC

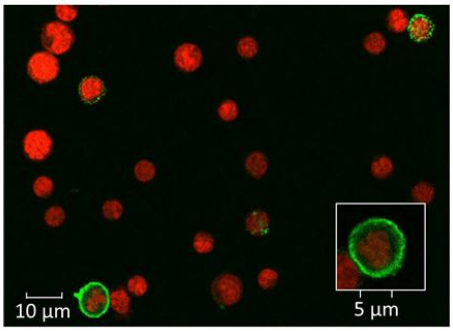

28.0 ± 6.6
345 ± 166.7
3301.8 ± 931.0
Count
CD45 clone F10-89-4

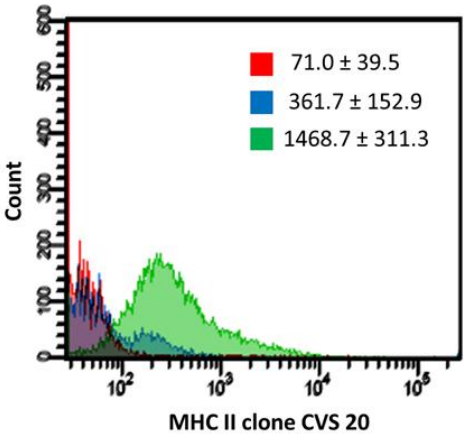

10 µm
5 µm
equine MNC

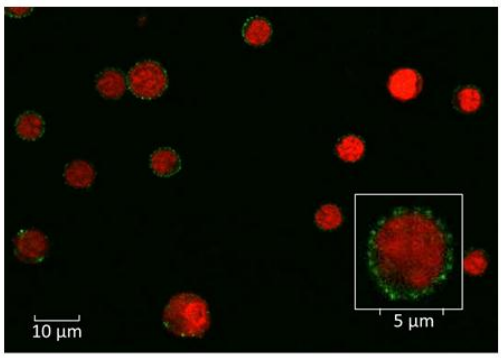

131.3 ± 119.5
677.3 ± 100.7
1945.3 ± 886.4
Count
CD79α clone HM57

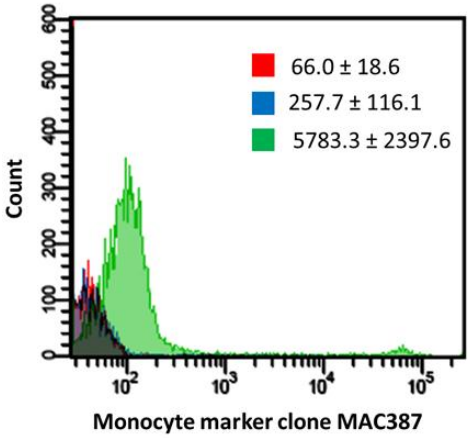

10 µm
5 µm
equine MNC

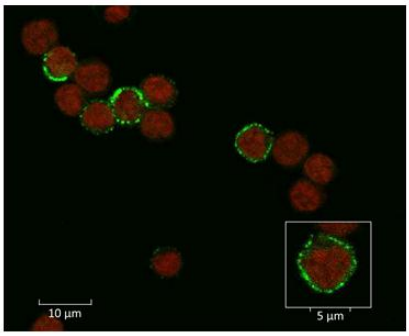

25.3 ± 6.2
422.7 ± 109.2
5405.0 ± 2531.4
Count
CD90 clone DH24A


10 µm
5 µm
equine MNC


71.0 ± 39.5
361.7 ± 152.9
1468.7 ± 311.3
Count
MHC II clone CVS 20


10 µm
5 µm
equine MNC


66.0 ± 18.6
257.7 ± 116.1
5783.3 ± 2397.6
Count
Monocyte marker clone MAC387


10 µm
5 µm
equine MNC

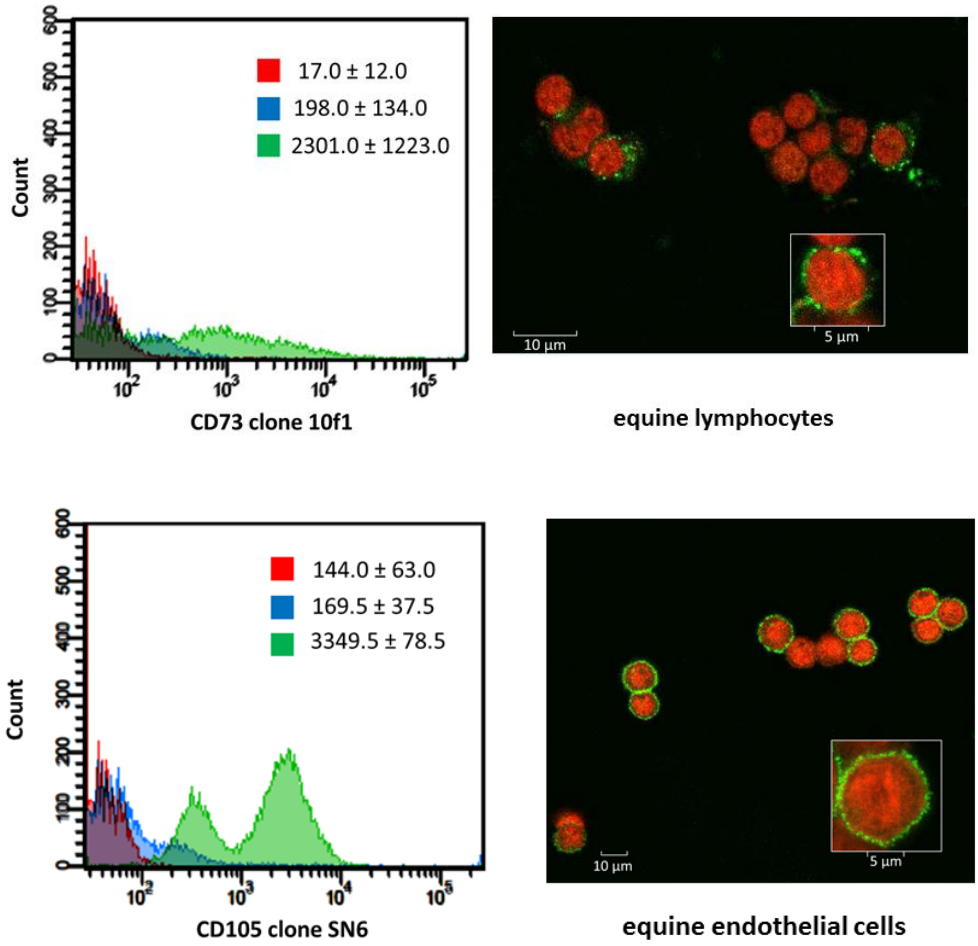


Figure 4. Flow cytometric and confocal fluorescence microscopic analyses of the cross-reacting monoclonal antibodies (mAbs) based on the appropriate equine positive control cells. Fluorescence channel histograms represent relative numbers of cells versus their mean fluorescence intensity (MFI). The red and blue histograms which systematically overlap, represent the negative controls (i.e. autofluorescence and relevant isotype control, respectively). The green histograms represent the test samples incubated with each of the selected mAbs. Mean MFI ± SEM values were described for each histogram. Isolated equine MNC were positive for CD29, CD44, CD45, CD79α, CD90, MHC II, and the monocyte marker, isolated equine lymphocytes were positive for CD73 and isolated equine endothelial cells were positive for CD105. To confirm the cellular binding of each cross-reacting mAbs on the appropriate positive control cells, confocal fluorescence microscopy was used. Nuclei are visualized using PI.

For the CD45 and CD73 markers, only one out of the five anti-human clones tested for each marker recognized the equine epitopes on MNC and lymphocytes, respectively (Fig. 4). However, the percentage of positive equine cells was rather low with on average 24.6% of the equine MNC being positive for CD45 and 16.8% of the lymphocytes being positive for CD73

(Fig. 4). A similar percentage was obtained when excluding the non-viable cells based on their 7-AAD positivity. Moreover, non-specific binding was minimalized by including appropriate blocking steps and negative controls such as relevant isotype controls for both mAbs. Still, to further evaluate the specificity of this positive signal, the anti-CD45 and anti-CD73 stained equine cells were visualized by confocal fluorescence microscopy. In addition, human MNC and lymphocytes were also used to analyze these two anti-human mAbs by flow cytometry as well as confocal fluorescence microscopy. Hereby, it was found that 65.3% of the human MNC expressed CD45 and 18.5% of the human lymphocytes stained positive for CD73 (Fig. 5). Since these percentages of positive cells are in the same range as those for their equine counterparts, it was concluded that these two clones cross-react with equine epitopes although further research like Western blot or immunoprecipitation analyses might be required to unambiguously confirm cross-reactivity.

Finally, to assess cross-reactivity of the CD34 and CD105 mAbs, pure populations of equine primary endothelial cells were used. One clone of the five CD105 mAbs tested identified the equine epitope (Table 1 and Fig. 4), while none of the five CD34 mAbs tested showed cross-reactivity (Table 1).

In conclusion, only 11 out of the 30 mAbs evaluated in the first part of this study recognized the respective equine epitopes, and as such, are useful to characterize equine MSC.

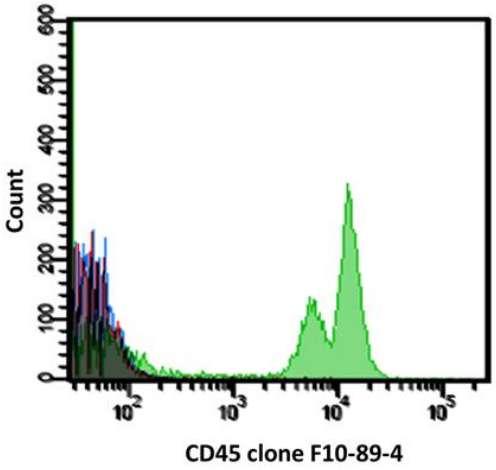


Fig. 2A

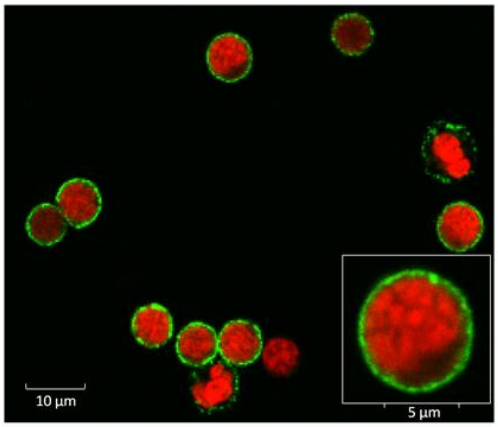


Fig. 2C

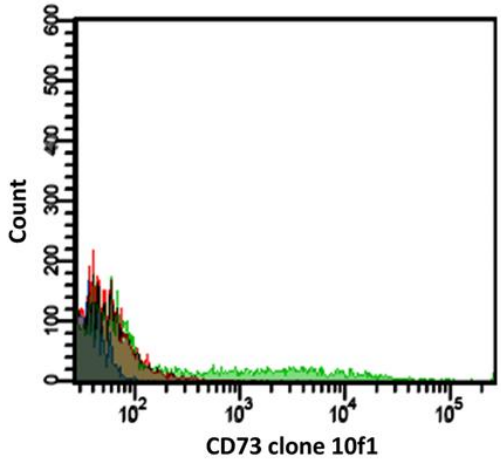


Fig. 2B

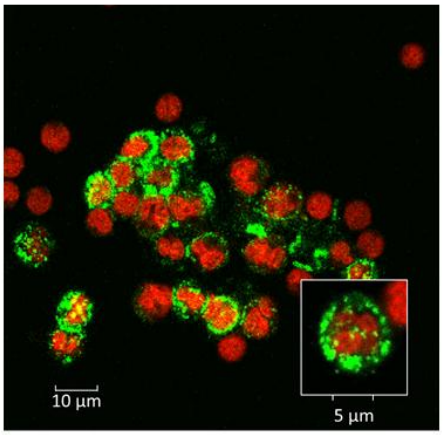


Fig. 2D

Figure 5. Flow cytometric and confocal fluorescence microscopic analyses of the cross-reacting mouse anti-human CD45-Alexa488 mAb on isolated human MNC, and of the mouse anti-human CD73 mAb on isolated human lymphocytes. Fluorescence channel histograms showing the expression of anti-human CD45 on isolated human MNC (A) and anti-human CD73 on isolated human lymphocytes (B), respectively. Histograms represent relative numbers of cells versus their mean fluorescence intensity (MFI). The red and blue histograms, which systematically overlap, represent the negative controls (i.e. autofluorescence and isotype control, respectively). The green histogram represents the test sample incubated with the mAb. To confirm the cell surface binding of the CD45mAb on the human MNC (C), and of the CD73 mAb on the human lymphocytes (D), confocal fluorescence microscopy was used. Nuclei are visualized using PI.

4.4.2. Immunophenotyping of equine UCB-derived MSC by multicolor flow cytometry

The cross-reacting mAbs identified in the first part of this study were used to immunophenotype equine MSC isolated from the UCB of six horses using multicolor flow cytometry, as outlined in Table 2. A representative example of the gating strategy and the multicolor analysis was shown in Figure 6. On average 92.8% [interquartile range (IQR) 90.6-94.7] of the undifferentiated MSC of the fourth passage simultaneously expressed CD29 and CD44 and lacked expression of MHC II (Table 3, subset 1). Also, on average 94.9% (IQR 89.6-99.6) MSC were positive for CD90, but had a low and variable expression of CD105 varying between 0.1 and 20.0% (Table 3, subset 2). The equine MSC lacked expression of CD45 (IQR 98.9-99.5) and displayed a variable expression for CD73 with the proportion of positive MSC ranging between 0.0 and 25.3% (Table 3, subset 3). Finally, on average 98.1% (IQR 97.2-99.1) of the MSC lacked expression of both CD79α and the monocyte marker (Table 3, subset 4). Equine MSC from the 10th passage as well as cryopreserved MSC from the fourth passage upon thawing, showed a virtually identical phenotype.

Table 3. Results of the immunophenotypic characterization of equine UCB-derived MSC from the fourth passage, expressed as the percentage (%) of cells either positive or negative for each of the selected 9 markers analyzed in 4 subsets [n=6; mean. median. standard error of the mean (SEM), interquartile range (IQR). minimum and maximum values]. Subset combinations are presented in bold.

marker	Mean	Median	SEM	IQR	Min	Max
$CD29^{pos}$	98.3	98.3	0.2	0.8	97.8	98.8
$MHC\ II^{neg}$	97.3	98.1	1.1	1.5	92	99.7
$7\text{-}AAD^{neg}$	97.1	97.2	0.7	1.4	94.2	98.9
$CD44^{pos}$	98.7	99	0.4	1.5	97.3	99.7
$CD29^{pos}$+$MHCII^{neg}$+$7\text{-}AAD^{neg}$+$CD44^{pos}$	**92.8**	**93**	**1.2**	**4.1**	**89.2**	**96.6**
$CD105^{pos}$	6	1.6	3.4	9.7	0.1	20
$7\text{-}AAD^{neg}$	89.9	88.5	2.8	7.7	80.3	99.5
$CD90^{pos}$	94.9	99.2	2.9	10.1	85	99.7
$CD105^{pos}$+$7\text{-}AAD^{neg}$+$CD90^{pos}$	**4.4**	**0.5**	**3**	**5**	**0.2**	**18.5**
$CD45^{neg}$	99.1	99.3	0.2	0.6	98.2	99.7
$CD73^{pos}$	4.6	0.4	4.1	1.1	0	25.3
$7\text{-}AAD^{neg}$	93.3	95.9	2.8	4.5	80.3	99.3
$CD45^{neg}$+$CD73^{pos}$+$7\text{-}AAD^{neg}$	**3.6**	**0.1**	**3.5**	**0.6**	**0**	**20.9**
$Monocyte^{neg}$	99.2	99.4	0.2	0.8	98.4	99.7
$CD79\alpha^{neg}$	98.7	99.4	0.5	1.9	96.9	99.7
$Monocyte^{neg}$+$CD79\alpha^{neg}$	**98.1**	**98.6**	**0.6**	**1.9**	**95.9**	**99.3**

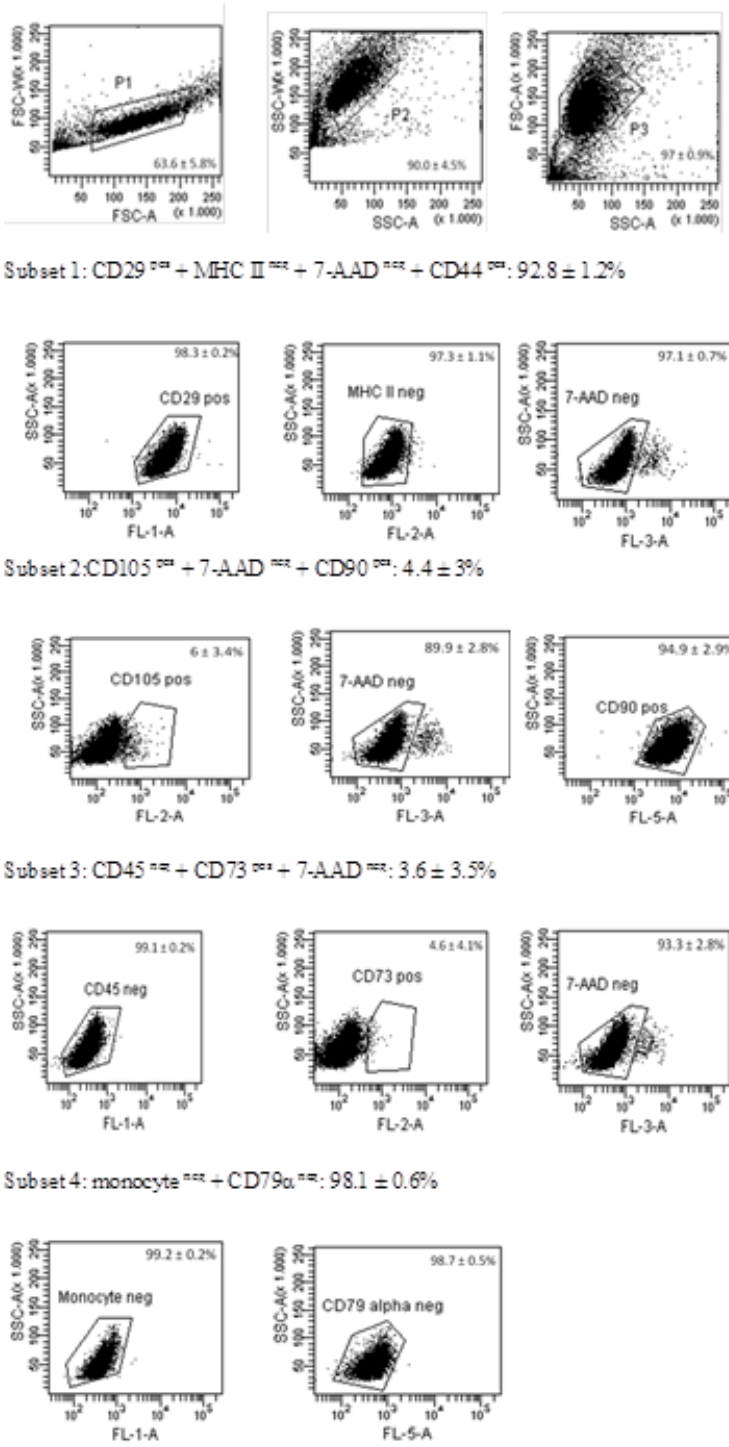


Figure 6. Gating strategy to enumerate the equine MSC. After visualizing the population of interest on the FSC-A/FSC-W dot plot (P1), P1 was gated on the SSC-A/SSC-W dot plot to discriminate for doublets and clumps (P2). Subsequently, the single cell population was identified by defining P2 on a SSC-A/FSC-A signal dot plot (P3). The final gate for analysis was a Boolean gate on the single cell population and the 7-AAD^{neg} cells, enabling the analysis of a viable single cell population. For each subset, this viable single cell population was displayed on the respective fluorescence channel vs SSC-A dot plots. Mean ± SEM values were described for each histogram.

4.5. Discussion

The unequivocal immunophenotyping of equine cells in general and MSC in particular, is strongly hampered by the limited availability of mAbs directed against equine epitopes or against epitopes of other species which show cross-reactivity with the horse. In a study of Ibrahim et al. (2007), only 14 out of the 379 tested anti-human mAbs, i.e. less than 5%, recognized the corresponding epitopes on isolated equine leukocytes. This illustrates the urgent need for additional studies evaluating potential cross-reactivity of xenogenic mAbs against specific molecules present on equine cells. In the present study, 30 mAb clones directed against epitopes used as markers to immunophenotype human MSC, were at first evaluated (Dominici et al., 2006). Eleven clones showed cross-reactivity with equine epitopes using MNC, lymphocytes or endothelial cells as positive control cells. Based on the cross-reacting mAb clones identified in the first part of this study, a multicolor marker panel based flow cytometric protocol to immunophenotype equine MSC was subsequently developed. Multicolor detection is an attractive strategy for the identification of MSC since different antigens on a single cell can be simultaneously detected (Martins et al., 2009) and the overlapping patterns of the phenotypic markers allow the discrimination of MSC from other cells (Tarnok et al., 2010). Some arguments were considered when composing the different subsets of the multicolor application. As it is interesting to identify the cells as simultaneously positive or negative for certain markers, following markers were combined: $CD29^{pos}$ and $CD44^{pos}$, $CD90^{pos}$ and $CD105^{pos}$, monocyte $marker^{neg}$ and $CD79\alpha^{neg}$. The combination in subset 4 was chosen from a practical point of view since these two mAbs detect intracellular antigens. For markers which require the most sensitivity, for example markers identifying dimly positive populations or when positive cells are very rare, bright fluorochromes such as PE, PE tandems or APC should be selected (Maecker et al., 2004; Mahnke & Roederer, 2007). It is indeed more likely that a bright fluorochrome will help to reveal the expression of

a given marker with a lower expression level (Baumgarth & Roederer, 2000). Moreover, if the expression level of the surface molecule is unknown, these bright fluorochromes are preferred as well (Baumgarth & Roederer, 2000). As such, most of the negative or dimly positive markers, i.e. MHC II, CD73, CD105 and CD79α, were combined with PE or APC in this study.

Although gene expression is also often assessed to evaluate the presence or absence of selected markers and is a complementary parameter to antigen measurement, mRNA is not always translated. Therefore, it is preferable to examine the presence or absence of the corresponding proteins rather than their mRNA levels (Guest et al., 2008).

The rationale for using the macrophage/monocyte anti-human mAb in this study, instead of the frequently used CD11b or CD14 clones, was based on the fact that its cross-reactivity with equine epitopes had been previously reported (Perez et al., 1999). This mAb recognizes the intracytoplasmic calprotectin molecule L1, which has a restricted distribution within the monocyte-derived cell lineage (Brandtzaeg et al., 1988). For CD34, we were not able to confirm the cross-reactivity of any of the five tested clones on equine endothelial cells although two of these clones were recently used in other studies to characterize equine MSC (Hoynowski et al., 2007; Martinello et al., 2010; Marfe et al., 2012). These apparently contradictory data emphasizes again the importance of using proper positive and negative control cells when evaluating cross-reactivity of mAbs. For example, no cross-reactivity experiments were described in the above mentioned studies, despite the fact that it was unsure whether the anti-human (Marfe et al., 2012) or anti-mouse (Martinello et al., 2010) mAbs were recognizing the equine epitopes.

As revealed in this study, viable equine MSC simultaneously expressed CD29 and CD44, and lacked expression of MHC II. They also simultaneously lacked expression of CD79α and the

monocyte marker. Due to the more variable expression of CD73 and CD105 between the six mares, less straightforward results were obtained for the combined expression of both these markers. Regardless, equine MSC clearly express CD90 and lack expression of CD45.

According to the ISCT criteria, the expression of CD105 on human MSC must exceed 95% (Dominici et al., 2006). In the present study, however, a low and variable expression was noted for UCB-derived equine MSC. Still, these results are valuable since it has been described in independent studies that human MSC isolated from UCB show a lower expression of CD105 (Maurice et al., 2006; Martins et al., 2009; Jenhani et al., 2011). Moreover, canine MSC isolated from adipose tissue, were recently reported to even be $CD105^{neg}$ (Vieira et al., 2010). These are all indications for a variable CD105 expression on MSC originating from either different sources and/or different species. For equine MSC, only Braun et al. (2010) have investigated CD105 using the same clone as the one used in the current study, for which they reported a strong positive signal. However, equine MSC were derived from adipose tissue instead of UCB in the latter study, which might explain the marked difference in observed CD105 expression. On the other hand, the detaching agent used could also provide an explanation for the apparent discrepancy. Indeed, in the study of Braun et al. (2010), accutase was used while trypsin-EDTA was used in our study. Trypsin is a pancreatic serine protease while accutase exhibits protease and collagenolytic activities (Hughes et al., 2009). Interestingly, it has been reported that trypsin can cause removal and/or functional impairment of certain cell-surface membrane proteins (Bryniarski et al., 2003; Mateusen et al., 2007). In line with these findings, Hackett et al. (2011) recently demonstrated that detaching equine cells with trypsin damaged certain cell surface proteins like CD14 while other markers such as CD90 appeared unaffected. Further research remains relevant to identify which equine epitopes are trypsin-labile.

Similar to CD105, the expression of CD73 on human MSC must exceed 95% according to the ISCT (Dominici et al., 2006). Nevertheless, a moderate expression has been reported for human MSC which were derived from bone marrow and cultured in medium containing FCS (Turnovcova et al., 2009). In line with the data reported for CD105 on canine MSC, these cells also appear to be $CD73^{neg}$ (Vieira et al., 2010). As the expression of CD73 has not been reported yet for equine MSC, it is difficult to compare our results with other studies or to provide an explanation for the variable CD73 expression observed in the current study.

In conclusion, this is the first report which describes a protocol to immunophenotype equine MSC isolated from UCB using multicolor flow cytometry. Hereby, the salient findings were that the equine MSC were $CD29^{pos}$, MHC II^{neg}, $CD44^{pos}$, $CD45^{neg}$, $CD90^{pos}$, $CD79\alpha^{neg}$ and monocyte $marker^{neg}$. The intriguing variability in expression of CD73 and CD105 on equine MSC, which is not in accordance with human MSC, warrants further research including potentially critical factors such as the influence of the source of equine MSC and the sample pretreatment. Furthermore, the application of this proposed multicolor protocol as an isolation tool to sort putative equine MSC from a mixed cell population, warrants further study as this would provide a major added value to this exciting research field. After all, the cultivation step required to identify the MSC based on their platic-adherency, can be circumvented as such.

4.6. Acknowledgements

The authors would like to thank Jella Wauters and Dries Vercauteren for the technical assistance with the (confocal) fluorescence microscopy. This research was partly funded by a feasibility study of the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, grant n° 1004132).

References

Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. J Immunol Meth 2000;243:77-97.

Brandtzaeg P, Jones DB, Flavell DJ, Fagerhol MK. Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. J Clin Pathol 1988;41:963-970.

Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, Walliser U, Skutella T, Aicher WK. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. Am J Vet Res 2010;71:1228-1236.

Bryniarski K, Maresz K, Szczepanik M, Ptak M, Ptak W. Modulation of macrophage activity by proteolytic enzymes. Differential regulation of IL-6 and reactive oxygen intermediates (ROIs) synthesis as a possible homeostatic mechanisms in the control of inflammation. Inflammation 2003;27:333-340.

De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011a;75:1431-1443.

De Schauwer C, Meyer E, Cornillie P, De Vliegher S, Van de Walle GR, Hoogewijs M, Declercq H, Govaere J, Demeyere K, Cornelissen M, Van Soom A. Optimization of the isolation, culture and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng Part C 2011b; 17(11):1061-1070.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.

Fortier LA, Travis AJ. Stem cells in veterinary medicine. Stem Cell Res Ther 2011;2:9.

Guest DJ, Ousey JC, Smith MRW. Defining the expression of marker genes in equine mesenchymal stromal cells. Stem Cells Cloning 2008;1:1-9.

Hackett CH, Flaminio MJBF, Fortier LA. Analysis of CD14 expression levels in putative mesenchymal progenitor cells isolated from equine bone marrow. Stem Cells Dev 2011;20:721-735.

Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T, Mitchell KE. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem Biophys Res Commun 2007;362:347-353.

Hughes OR, Stewart R, Dimmick I, Jones EA. A critical appraisal of factors affecting the accuracy of results obtained when using flow cytometry in stem cell investigations: where do you put your gates? Cytometry part A 2009;75A:803-810.

Ibrahim S, Saunders K, Kydd JH, Lunn DP, Steinbach F. Screening of anti-human leukocyte monoclonal antibodies for reactivity with equine leukocytes. Vet Immunol Immunopathol 2007;119:63-80.

Jenhani F, Durand V, Ben Azouna N, Thallet S, Othmen B, Bejaoui M, Domenech J. Human cytokine expression profile in various conditioned media for *in vitro* expansion bone marrow and umbilical cord blood immunophenotyped mesenchymal stem cells. Transplant. Proc. 2011;43:639-643.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

MacEachern KE. Methods for the isolation, culture and characterization of equine pulmonary artery endothelial cells. Res Vet Sci 1997;62:147-152.

Maecker HT, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. Cytometry part A 2004;62A:169-173.

Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med 2007;27:469-485.

Marfe G, Massaro-Giordano M, Ranalli M, Cozzoli E, Di Stefano C, Malafoglia V, Polettini M, Gambacurta A. Blood derived stem cells: an ameliorative therapy in veterinary ophthalmology. J Cell Physiol 2012;227(3):1250-1256.

Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M. Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood. Tissue Eng Part C 2010;16:771-781.

Martins AA, Paiva A, Morgado JM, Gomes A, Pais ML. Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplant. Proc 2009;41:943-946.

Mateusen B, Van Soom A, Maes DGD, Favoreel H, Nauwynck HJ. Receptor-determined susceptibility of preimplantation embryos to pseudorabies virus and porcine reproductive and respiratory syndrome virus. Biol Reprod 2007;76:415-423.

Maurice S, Srouji S, Livne E. Isolation of progenitor cells from cord blood using adhesion matrices. Cytotechnology 2006;52:125-137.

Pacini S, Spinabella S, Trombi L, Fazzi R, Galimberti S, Dini F, Carlucci F, Petrini M. Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses. Tissue Eng 2007;13:2949-2955.

Perez J, Mozos E, Martin MP, Day MJ. Immunohistochemical study of the inflammatory infiltrate associated with equine squamous cell carcinoma. J Comp Path 1999;121:385-397.

Radcliffe CH, Flaminio MJBF, Fortier LA. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev 2010;19:269-281.

Reed SA, Johnson SE. Equine umbilical cord blood contains a population of stem cells that express Oct4 and differentiate into mesodermal and endodermal cell types. J Cell Physiol 2008;215:329-336.

Richardson LE, Dudhia J, Clegg PD, Smith R. Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury. Trends Biotechnol 2007;25:409-416.

Strober W. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 2001 Appendix 3, Appendix 3B.

Tarnok A, Ulrich H, Bocsi J. Phenotypes of stem cells from diverse origin. Cytometry Part A 2010;77A:6-10.

Turnovcova K, Ruzickova K, Vanecek V, Sykova E, Jendelova P. Properties and growth of human bone marrow mesenchymal stromal cells cultivated in different media. Cytotherapy 2009;11:874-885.

Van de Walle GR, Peters ST, Van der Ven BC, O'Callaghan DJ, Osterrieder N. Equine herpesvirus 1 entry via endocytosis is facilitated by alpha V integrins and an RSD motif in glycoprotein D. J Virol. 2008;82(23):11859-11868.

Vieira NM, Brandalise V, Zucconi E, Secco M, Strauss BE, Zatz M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 2010;19:279-289.

Xie W, Wang X, Du W, Liu W, Qin X, Huang S. Detection of molecular targets on the surface of $CD34^{+}CD38^{-}$ bone marrow cells in myelodysplastic syndromes. Cytometry part A 2010;77A:840-848.

CHAPTER 5

SUCCESSFUL ISOLATION OF EQUINE MESENCHYMAL STROMAL CELLS FROM CRYOPRESERVED UMBILICAL CORD BLOOD-DERIVED MONONUCLEAR CELLS.

De Schauwer C, Van de Walle GR, Piepers S, Hoogewijs MK, Govaere JLJ, Meyer E[*]*, Van Soom A*[*]*. Succesful expansion of equine mesenchymal stromal cells from cryopreserved umbilcal cord blood-derived mononuclear cell fractions. Equine Veterinary Journal 2012, in press.*

[*]*shared senior authorship*

5.1. Abstract

The therapeutic potential of mesenchymal stromal cells (MSC) for cellular therapy has generated an increasing interest in human as well as in veterinary medicine. Considerable research has been performed on the cryopreservation of expanded MSC, but little information is available on the cryopreservation of the original mononuclear cell fraction (MNC). The present study describes a protocol to expand equine MSC after cryopreserving the MNC of umbilical cord blood (UCB). To this end, MNC were isolated from seven UCB samples and cryopreserved at a concentration of $1\text{-}2\times10^6$ cells/mL cold freezing solution. Cells were cryopreserved for at least six months before thawing. Frozen cryotubes were thawed in a 37°C water bath. Putative equine MSC were immunophenotyped using multicolor flow cytometry based on a selected 9-marker panel. Average cell viability upon thawing was 98.7 ± 0.6 %. In 6 out of 7 samples, adherent spindle-shaped cell colonies were observed within 9.0 ± 2.6 days and attained 80% confluency at 12.3 ± 3.9 days. After three passages, putative equine MSC were successfully immunophenotyped as CD29, CD44, and CD90 positive, and CD45, CD73, CD79α, CD105, MHC II and monocyte-marker negative. Equine MSC can be cultured after cryopreservation of the isolated MNC, a time- as well as cost-efficient approach in equine regenerative medicine.

5.2. Introduction

Mesenchymal stromal cells (MSC) are adult stem cells which are theoretically considered multipotent as they can only differentiate into organ-specific cell types of the mesodermal somatic germ layer. Nevertheless, recent studies illustrate the differentiation potential of these MSC into cell types of tissue lineages different from the tissue of origin, giving rise to the concept of stem cell plasticity (Fortier, 2005). As such, these cells can be

used as a promising tool in regenerative medicine, which has generated an increasing interest in both human and veterinary medicine (Casado-Diaz et al., 2008).

Mesenchymal stromal cells are characterized by their ability to adhere to plastic and by their capacity of multipotent differentiation, i.e. towards the osteogenic, chondrogenic and adipogenic lineage. Furthermore, they must express a panel of MSC markers such as CD29, CD44, CD73, CD90, and CD105 while lacking distinctive hematopoietic antigens like CD34, CD45, CD14, CD79α and MHC II (Dominici et al., 2006).

The cryopreservation of equine MSC is described previously without signifantly impairing their morphology, proliferation potential and differentiation capacities after post-thawing expansion (Koch et al., 2007; Casado-Diaz et al., 2008). Interestingly, only a few reports in human medicine describe the isolation of MSC after cryopreserving the mononuclear cells (MNC) (Lee et al., 2004; Kögler et al., 2005; Kögler et al., 2006; Casado-Diaz et al., 2008) and only one study so far has evaluated the sample sterility, as well as the cell recovery and viability after thawing processed equine UCB (Shuh et al., 2009). Since the use of both autologous and allogeneic MSC for treatment of various diseases has evolved rapidly in recent years, the concept of UCB banking for future use has received particular attention in human medicine (Lee et al., 2004), and may be equally applicable for equine medicine.

The major aim of the present study was therefore to evaluate the potential of equine MSC isolation from cryopreserved umbilical cord blood (UCB)-derived MNC. Subsequently, the putative equine MSC were immunophenotyped using multi-color flow cytometry to confirm their mesenchymal identity.

5.3. Materials & Methods

5.3.1. Collection and processing of equine umbilical cord blood

Before the umbilical cord ruptured spontaneously, UCB was collected from 7 foals immediately after parturition, with informed consent of the owner. The umbilical cord was clamped as closely as possible to the vulva of the mare, as such avoiding any close contact with the contaminated external environment of the stable. Subsequently, the umbilical cord was disinfected with 70% alcohol, using two different swipes. The cord was only punctured once. Umbilical cord blood was drained by gravity into a sterile standard 350-mL blood donor bag containing 49 mL CPD A anticoagulant (Terumo[a]). Every sample was processed within 15 hours after collection, as previously described (De Schauwer et al., 2011). The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2010/147).

5.3.2. Cryopreservation and subsequent culture of isolated equine mononuclear cells

Equine MNC were isolated from 7 UCB samples using a Percoll® gradient (density 1.080 – GE Healthcare, Little Chalfont, United Kingdom), and cryopreserved at a concentration of 1-2×10^6 cells per mL cold freezing solution consisting of high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Gent, Belgium), 10% fetal calf serum (Gibco, Gent, Belgium) and 20% dimethylsulphoxide (Sigma, Bornem, Belgium), as described by Koch et al. (2007). Cells were cryopreserved using a programmable freezer (IceCube 14S, Sylab, Vienna, Austria) using following freezing curve: from 4°C until -70°C, temperature decreased with 1°C/min and from -70°C until -140°C with 10°C/min (Zerbe et al., 2009). Cells were stored in liquid nitrogen for at least 6 months before thawing. Frozen cryotubes were thawed in a 37°C wather bath and immediately thereafter transferred to 6 mL of equilibrated medium. After centrifuging 200 ×g for 10 min at RT, supernatant was removed and the cell pellet was resuspended in 3 mL equilibrated medium. Cell concentration and viability were determined

using Trypan blue exclusion before incubation at 37.5°C in a humidified atmosphere containing 5% CO_2. The culture medium contained low-glucose DMEM (Invitrogen, Gent, Belgium), 30% fetal calf serum (Gibco, Gent, Belgium), 10^{-7} M low dexamethazone, 50 µg/mL gentamicin, 10 µl/mL antibiotic antimycotic solution, 250 ng/mL fungizone (all from Sigma, Bornem, Belgium) and 2 mM ultraglutamine (Invitrogen, Gent, Belgium). Non-adherent cells were removed after overnight incubation by replacing the culture medium. Remaining non-adherent cells were removed by exchanging the culture medium twice a week. Cultures were inspected every day for the presence of adherent spindle-shaped cells. As soon as confluency exceeded 80%, cells were trypsinized using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma, Bornem, Belgium) and cultured in expansion medium which was identical to the culture medium except for dexamethasone (Koch et al., 2007).

5.3.3. Immunophenotyping of cultured equine mesenchymal stromal cells

For the immunophenotyping experiments, cryopreserved and thawed undifferentiated equine MSC from the third or fourth passage were used. The multicolor flow cytometry was performed using the same marker panel combinations as previously described (De Schauwer et al., 2012).

At least 10,000 cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems, Erembodegem, Belgium) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser. All data were corrected for autofluorescence as well as for unspecific bindings using both secondary Ab and/or isotype negative controls. Compensation for spectral overlap between fluorochromes was performed using an automatic calibration technique (FACSDiva software, Becton Dickinson, Erembodegem, Belgium).

5.3.4. Gating strategy

A primary gate was placed on the area versus width signal of the forward scatter (FSC-A/FSC-W) dot plot, after which this population was visualized on the area versus width signal of the side scatter (SSC-A/SSC-W) dot plot to discriminate for doublets and clumps. Subsequently, the gated population as demonstrated on a side scatter area signal versus a forward scatter area (SSC-A/FSC-A) signal dot plot, defined the single cell population. Finally, a Boolean gate on the single cell population and the 7-AADneg cells enabled the analysis of a viable single cell population.

5.3.5. Statistical analysis

All data were analyzed using FACSDiva software (Becton Dickinson, Erembodegem, Belgium). The difference in the proportion of MSC positive for the different tested cell markers between freshly isolated MSC and cryopreserved MSC was evaluated using the non-parametric independent samples Mann-Whitney U test (SPSS Statistics 19.0). The difference in the time until the first passage between both groups of MSC was tested using the independent samples T-test (SPSS Statistics 19.0). Data are presented as mean ± standard deviation. Statistical difference was assessed at $P < 0.05$.

5.4. Results

After thawing of cryopreserved equine MNC from 7 UCB samples, cell viability was 98.7 ± 0.6 %. In 6 out of 7 samples, adherent spindle-shaped cell colonies, the characteristic morphology for MSC, occurred within 9 ± 2.6 days of culturing in MSC medium while no MSC colonies were isolated in one sample. Cultures were 80% confluent at 12.3 ± 3.9 days (Fig. 1). These data were not significantly different from the data that were obtained from directly isolated and cultured equine MSC from the same UCB samples, using the same

protocol, i.e. 7.7 ± 1.7 days and 15.1 ± 2.8 days (*P*=0.139 and *P*=0.293, respectively) (unpublished data).

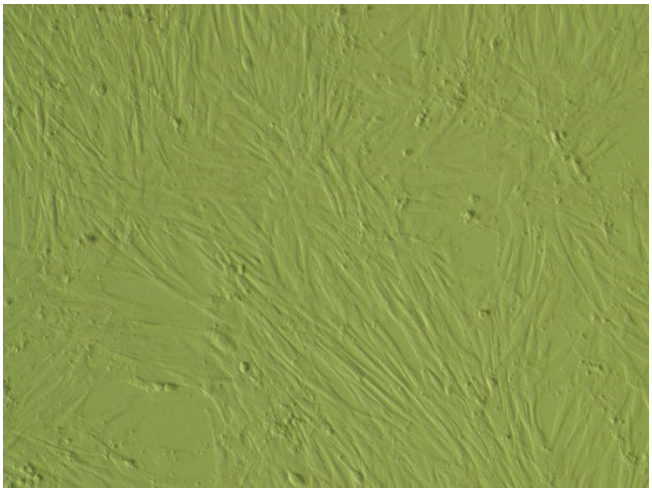


Figure 1. Representative light microscopic picture of adherent spindle-shaped equine MSC isolated from cryopreserved MNC (60×).

After three passages, the putative equine MSC were successfully immunophenotyped using multi-color flow cytometry based on a selected panel of markers (Table 1) (De Schauwer et al., 2012). Indeed, cells were positive for CD29, CD44, and CD90, and negative for CD45, CD73, CD79α, CD105, MHC II and the monocyte marker (Fig. 2). On average 93.9 ± 2.9 % of the viable MSC expressed simultaneously CD29 and CD44, and lacked expression of MHC II (subset 1 of markers), corroborating the data of isolated MSC from freshly collected equine UCB (*P*=0.537) (De Schauwer et al., 2012). For subset 2 of markers, 98.5 ± 1.6 % of the MSC were positive for CD90 while CD105 was not detectable. Again, these data were not significantly different when compared to equine MNC which were immediately cultured (*P*=0.537 and *P*=0.126) (De Schauwer et al., 2012). Furthermore, 99.7 ± 0.1 % of the isolated viable MSC were negative for both CD45 and CD73 (marker subset 3). For the fourth marker subset combination, 95.0 ± 5% of the undifferentiated MSC were negative for both the monocyte marker and CD79α, which again was similar to data obtained

for freshly isolated MSC (*P*=0.329) (De Schauwer et al., 2012). The only significant immunophenotypical difference observed between freshly isolated MSC and MSC cultured from cryopreserved MNC, was at the level of CD45 (*P*=0.017); 99.1 ± 0.5% of the former were CD45 negative (De Schauwer et al., 2012), whereas for the latter, 99.7 ± 0.1 % were CD45 negative (Fig. 2).

Table 1. Immunophenotypic characterization of equine mesenchymal stromal cells from umbilical cord blood-derived cryopreserved mononuclear cells, expressed as the percentage (%) of cells either positive or negative for each of the 9 markers analyzed in 4 subsets [n=6; mean, standard deviation (St Dev), minimum and maximum values]. Subset combinations are presented in bold.

subset	marker	Mean	St Dev	Min	Max
	$CD29^{pos}$	99.5	0.3	99.2	99.8
	$MHC\ II^{neg}$	99.5	0.2	99.3	99.8
	$7\text{-}AAD^{neg}$	95.6	2.8	91.6	98.2
	$CD44^{pos}$	97.7	2.7	93.2	99.8
1	**$CD29^{pos}$+$MHCII^{neg}$+$7\text{-}AAD^{neg}$+$CD44^{pos}$**	**93.9**	**2.9**	**90.3**	**97.5**
	$CD105^{pos}$	0.3	0.2	0.1	0.6
	$7\text{-}AAD^{neg}$	96.4	2.6	92.2	99.1
	$CD90^{pos}$	98.5	1.7	96.6	99.8
2	**$CD105^{pos}$+$7\text{-}AAD^{neg}$+$CD90^{pos}$**	**0.2**	**0.1**	**0.1**	**0.4**
	$CD45^{neg}$	99.7	0.2	99.5	99.9
	$CD73^{neg}$	99.7	0.4	99.0	99.9
	$7\text{-}AAD^{neg}$	95.8	3.9	89.4	99.2
3	**$CD45^{neg}$+$CD73^{neg}$+$7\text{-}AAD^{neg}$**	**99.7**	**0.4**	**99.0**	**99.9**
	$Monocyte^{neg}$	99.5	0.3	99.2	99.8
	$CD79\alpha^{neg}$	95.4	5.1	87.1	99.5
4	**$Monocyte^{neg}$+$CD79\alpha^{neg}$**	**95.0**	**5.0**	**86.7**	**99.2**

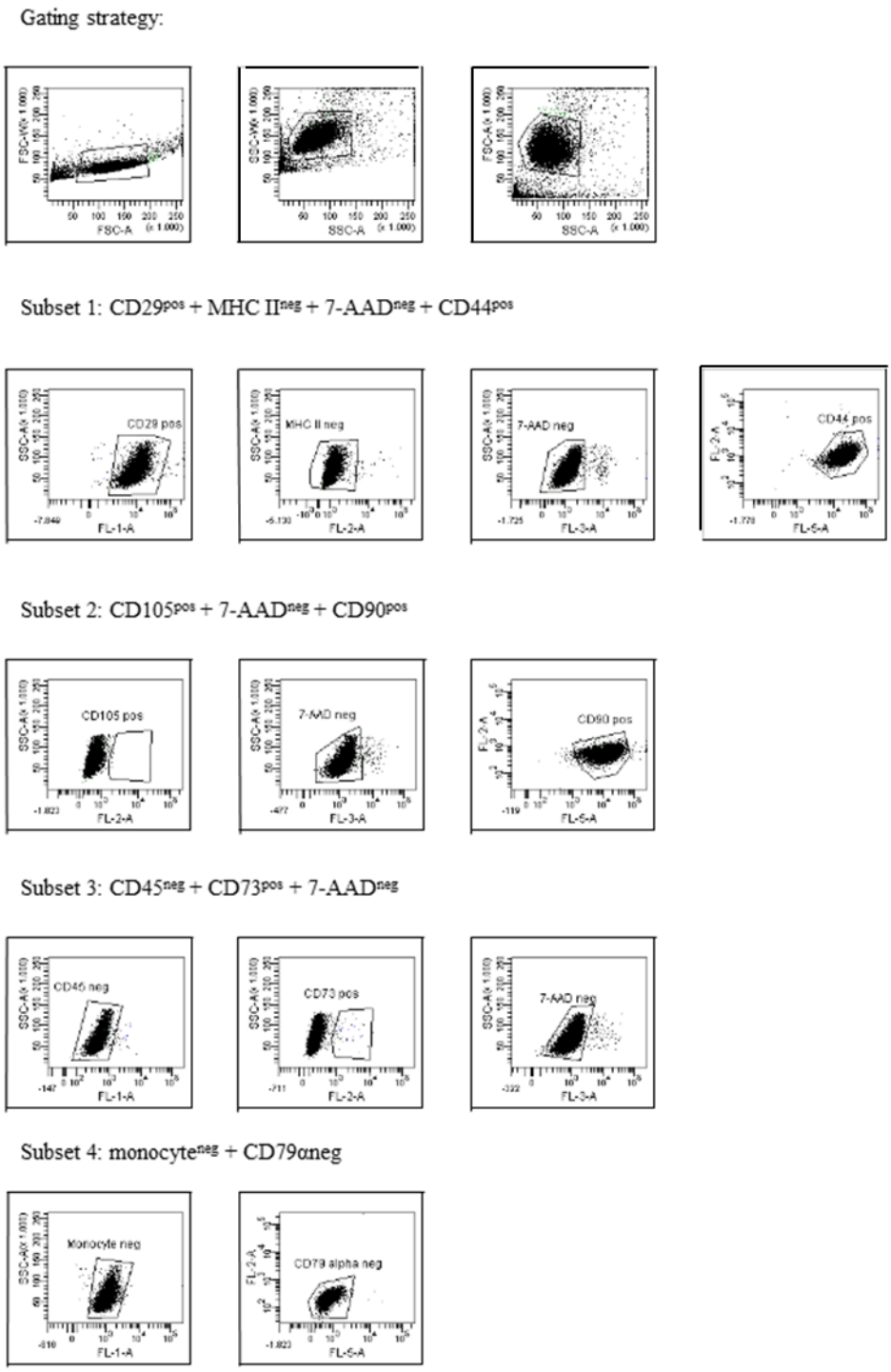


Figure 2. Representative dot-plots of one sample demonstrating the gating strategy as well as the immunophenotypic characterization of equine mesenchymal stromal cells isolated from the cryopreserved mononuclear cell fraction. After visualizing the population of interest on the FSC-A/FSC-W dot plot (P1), P1 was gated on the SSC-A/SSC-W dot plot to discriminate for doublets and clumps (P2). Subsequently, the single cell population was identified by defining P2 on a SSC-A/FSC-A signal dot plot (P3). The final gate for analysis was a Boolean gate on the single cell population and the 7-AAD^{neg} cells, enabling the analysis of a viable single cell population. For each subset, this viable single cell population was displayed on the respective fluorescence channel vs SSC-A dot plot. Data were represented as mean ± standard deviation.

5.3. Discussion

The successful expansion of MSC from cryopreserved equine MNC holds a significant advantage because MNC can be obtained from UCB and cryopreserved within a couple of hours. As such, cryopreserved MNC can be stored to isolate MSC for future research and/or clinical applications. This strategy avoids the use of a time-consuming work-up protocol requiring both equipment and experienced personnel to process the UCB and start the cultures upon collection (Casado-Diaz et al., 2008).

The immunophenotypic profile of freshly isolated MSC and MSC cultured from cryopreserved MNC was virtually identical, with one exception, namely a statistically significant difference for the expression of CD45. Since there was only a difference of 0.6% in CD45 expression between both groups (99.1 ± 0.5 % versus 99.7 ± 0.2 %), this difference is most likely not of any biological relevance. However, due to the minimal variation between the different samples, as indicated by the very small standard deviations (0.5% and 0.2%, respectively), this difference turned out to be statistically significant. The lack of any detectable expression of CD73 and CD105 in the present study was similar to what has been found previously (De Schauwer et al., 2012) and can be explained as follows. Although it has been stated by the International Society of Cellular Therapy that MSC should be more than 95 % positive for CD73 and CD105, several studies report a lower or even no expression for human, canine or equine MSC (Maurice et al., 2006; Martins et al., 2009; Turnovcova et al., 2009; Vieira et al., 2010; Jenhani et al., 2011; Pascucci et al., 2011). Furthermore, it has been demonstrated that certain cell surface markers can be trypsin-labile which causes a functional impairment or even their removal (Hackett et al., 2011). If this applies for CD73 and CD105, MSC would appear negative for markers upon immunophenotyping.

In the current study, a functional characterization by means of differentiation experiments was not performed based on following rationale: (i) UCB-derived MSC were previously functionally characterized by a successful differentiation towards the osteogenic, chondrogenic and adipogenic lineage, using the exact same isolation protocol as employed in the current study (De Schauwer et al., 2011) and (ii) it has been stated by Dominici et al. (2006) that every cell preparation of MSC isolated using a given protocol does not need to be re-evaluated.

Although it has been previously suggested that it is difficult to isolate and expand equine MSC from cryopreserved UCB (Koch et al., 2007), equine MSC were successfully isolated in 6 out of 7 cryopreserved UCB-derived MNC samples in the present study, using the protocol which was described previously (De Schauwer et al., 2011). This presents new opportunities since MNC samples from equine UCB samples can be cost- and time-efficiently banked for possible autologous or allogeneic therapeutic use in the future.

5.5.Acknowledgements

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References

Casado-Diaz A, Santiago-Mora R, Jimenez R, Caballero-Villarraso J, Herrera C, Torres A, Dorado G, Quesada-Gomez JM. Cryopreserved human bone marrow mononuclear cells as a source of mesenchymal stromal cells: application in osteoporosis research. Cytotherapy 2008;10:460-468.

De Schauwer C, Meyer E, Cornillie P, De Vliegher S, Van de Walle GR, Hoogewijs M, Declercq H, Govaere J, Demeyere K, Cornelissen M, Van Soom A. Optimization of the isolation, culture and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng Part C, 2011;17(11):1061-1070.

De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JLJ, Braeckmans K, Van Soom A, Meyer E. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry part A, 2012;81(4):312-323.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.

Fortier LA. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005;34(5): 415-423.

Hackett CH, Flaminio MJBF, Fortier LA. Analysis of CD14 expression levels in putative mesenchymal progenitor cells isolated from equine bone marrow. Stem Cells Dev 2011;20:721-735.

Jenhani F, Durand V, Ben Azouna N, Thallet S, Othmen B, Bejaoui M, Domenech J. Human cytokine expression profile in various conditioned media for *in vitro* expansion bone marrow and umbilical cord blood immunophenotyped mesenchymal stem cells. Transplant. Proc. 2011;43:639-643.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

Kögler G, Rade TF, Lefort A, Sensken S, Fischer J, Sorg RV, Wernet P. Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. Exp Hematol 2005;33:573-583.

Kögler G, Sensken S, Wernet P. Comparative generation and characterization of pluripotent unrestricted somatic stem cells with mesenchymal stem cells from human cord blood. Exp Hematol 2006;34:1589-1595.

Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC, Kim YJ. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochem Biophys Res Commun 2004;320:273-278.

Martins AA, Paiva A, Morgado JM, Gomes A, Pais ML. Quantification and immunophenotypic characterization of bone marrow and umbilical cord blood mesenchymal stem cells by multicolor flow cytometry. Transplant. Proc 2009;41:943-946.

Maurice S, Srouji S, Livne E. Isolation of progenitor cells from cord blood using adhesion matrices. Cytotechnology 2006;52:125-137.

Pascucci L, Gurina G, Mercati F, Marini C, Dall'Aglio C, Paternesi B, Ceccarelli P. Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: towards the definition of minimal stemness criteria. Vet Immunol Immunopathol 2011;144:499-506.

Shuh EM, Friedman MS, Carrade DD, Li J, Heeke D, Oyserman SM, Galuppo LD, Lara DJ, Walker NJ, Ferraro GL, Owens SD, Borjesson DL. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical-cord blood. Am J Vet Res 2009;70:1526-1535.

Turnovcova K, Ruzickova K, Vanecek V, Sykova E, Jendelova P. Properties and growth of human bone marrow mesenchymal stromal cells cultivated in different media. Cytotherapy 2009;11:874-885.

Vieira NM, Brandalise V, Zucconi E, Secco M, Strauss BE, Zatz M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 2010;19:279-289.

Zerbe H, Castilho LFF, Engelke F, Mattos RC, Schuberth H-J, Klug E, Leibold W. Isolation and cryopreservation of functionally competent equine leucocytes. J. Vet. Med. A 2003;50:179-184.

CHAPTER 6

COMPARATIVE ANALYSIS OF EQUINE MESENCHYMAL STROMAL CELLS ISOLATED FROM ALTERNATIVE SOURCES.

De Schauwer C, Van de Walle GR, Piepers S, Hoogewijs MK, Govaere JLJ, Meyer E, Van Soom A. Comparative analysis of equine mesenchymal stromal cells isolated from alternative sources. Journal of Tissue Engineering and Regenerative Medicine, submitted.

6.1. Abstract

In veterinary medicine, mesenchymal stromal cells (MSC) have been extensively studied the past ten years as their abilities in regenerative medicine are promising. Although bone marrow is the best known source for isolating MSC, the harvest of these cells is a highly invasive procedure. Umbilical cord blood (UCB), peripheral blood (PB) and umbilical cord matrix (UCM) are easily available sources of MSC and therefore, equine MSC obtained from these three origins were compared and following parameters were analyzed: success rate of isolating MSC, proliferation capacity, tri-lineage differentiation ability and immunophenotypic profile. Different sources were compared within each individual horse as matched samples were obtained at parturition. While equine MSC could be isolated from all the UCB and PB samples (6/6), a successful isolation was obtained in only 2 UCM samples. Proliferation data indicated that equine MSC from the three sources could be easily expanded, although UCB-derived MSC appeared significantly faster in culture than PB- or UCM-derived MSC. Equine MSC from both UCB and PB could be differentiated towards the osteogenic, chondrogenic and adipogenic lineage. For UCM-derived MSC, only chondrogenic and adipogenic differentiation could be confirmed. Differences in the levels of marker expression were statistically significant, though biologically not likely to be relevant. Based on these findings, it can be concluded that both UCB, and to a lesser extent PB, can be valuable alternatives for bone marrow and adipose tissue as a source of MSC.

6.2. Introduction

In the past decade, equine mesenchymal stromal cells (MSC) have received much attention as they are an attractive cell source for cell-based therapies given their ability to promote tissue regeneration and their immunomodulatory as well as anti-inflammatory

capacities (Borjesson & Peroni, 2011). Traditionally, bone marrow and adipose tissue have been used to harvest equine MSC. Alternative sources include umbilical cord blood (UCB), umbilical cord matrix (UCM), peripheral blood (PB), tendon, gingiva, periodontal ligament, and amniotic membrane or fluid (Smith et al., 2003; Koerner et al., 2006; Hoynowski et al., 2007; Koch et al., 2007; Stewart et al., 2009; Braun et al., 2010; Lange-Consiglio et al., 2011; Lovati et al., 2011; Mensing et al., 2011; Iacono et al., 2012).

Although bone marrow is the main source for isolating MSC, harvesting it is a highly invasive procedure with safety concerns for both the patient and the clinician (Berg et al., 2009). Potential drawbacks when isolating MSC from bone marrow are pain associated with the collection, hemorrhage, infection, pneumothorax, pneumopericardium and even pericardial laceration (Nixon et al., 2008; Brehm et al., 2012). The collection of adipose tissue is considered less invasive, but it can be difficult to obtain MSC in highly trained athletic horses because of the small amount of accessible fat (Carrade et al., 2011). Issues concerning ease of isolation, expansion characteristics and donor site complications suggest the search for alternative sources granted that the isolated MSC share the same characteristics as the bone marrow- or adipose tissue-derived MSC (Toupadakis et al., 2010). Mesenchymal stromal cells from UCB and UCM can be easily collected at parturition without harming the mare or the foal, expanded and subsequently cryopreserved, being readily available at the time of injury. Thus, the optimal time for treatment can be determined by the clinician which is in sharp contrast to the use of MSC from bone marrow or adipose tissue for which the time for cellular expansion must be taken into account (Berg et al., 2009). Nevertheless, the procedure of cryopreservation implies a long-term storage (Berg et al., 2009). Peripheral blood is also considered as an attractive alternative for bone marrow or adipose tissue since the collection of a venous blood sample is a minimal-invasive and easy procedure which can be performed at any time, unlike UCB and UCM (Martinello et al., 2010; Spaas et al., 2012). Therefore, the

aim of the present study was to compare these three attractive sources of equine MSC, i.e. UCB, PB and UCM, under identical *in vitro* conditions, to see whether they can be considered as a valuable alternative to bone marrow-derived MSC. To this end, following parameters were analyzed and compared: (i) success rate of isolating MSC, (ii) proliferation capacity, (iii) tri-lineage differentiation ability and (iv) immunophenotypical profile.

6.3. Materials and Methods

6.3.1. Collection of umbilical cord blood, umbilical cord matrix and peripheral blood

After clamping and disinfecting the umbilical cord with 70% alcohol, the umbilical vessel was punctured immediately after birth and UCB was drained by gravity into a standard blood donor bag (Terumo®), and subsequently stored at 4°C. Samples were only processed if they fulfilled the minimal criteria described for human UCB (Bieback et al., 2004), i.e. (i) at least 150 mL UCB was collected, (ii) storage time was less than 15 hours, and (iii) no signs of coagulation or hemolysis were present.

Once the umbilical cord was ruptured spontaneously, a clamp was placed on each end of the amniotic part after which the umbilical cord was rinsed with tab water and iodine soap to remove the gross contamination (Vidal et al., 2012), and disinfected with 70% alcohol. Subsequently, a 5-cm long piece was obtained from the middle of the disinfected umbilical cord with a sterile scalpel blade, and stored in phosphate buffered saline (PBS) containing 50 µg/mL gentamicin at 4°C.

At the same moment, PB from the *vena jugularis* was collected into two vacuum blood tubes containing heparin as anti-coagulant, and stored at 4°C until further processing.

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (EC2010/147).

6.3.2. Isolation and culture of equine mesenchymal stromal cells

Equine MSC derived from UCB and PB were isolated and cultured as previously described (De Schauwer et al., 2011). Briefly, UCB or PB was centrifuged at 1000 ×g for 20 min at room temperature (RT). After diluting the obtained buffy coat fraction 1:1 (v:v) with Hank's Balanced Salt Solution (HBSS), the cell suspension was gently layered on an equal volume of Percoll® (density 1.080 g/mL; GE Healthcare, Little Chalfont, United Kingdom) and centrifuged for 15 min at 600 ×g at RT. The interphase was collected and washed three times with HBSS by centrifuging 10 min at 200 ×g at RT. Cell viability and concentration was determined by trypan blue exclusion using the improved Neubauer hemocytometer. Isolated cells were seeded at 1×10^6 cells/mL in uncoated T-25 culture flasks and incubated at 37.5°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells were removed the following day by completely replacing the culture medium. Next, the culture medium was exchanged twice weekly. When numerous colonies of adherent cells were observed, the cells were detached using 0.083% trypsin-ethylenediaminetetraaceticacid (EDTA) (Sigma, Bornem, Belgium).

In a laminar flow hood, the umbilical cord was disinfected with Octeniderm® antiseptic spray (Schülke & Mayr) after which the umbilical arteries and vein were removed. The UCM was minced finely ($0.5cm^2$) using sterile scissors in a sterile glass Petri dish containing UCM culture medium. Subsequently, the explants were transferred to a T-25 culture flask in 6 mL UCM culture medium and incubated at 37.5°C in a humidified atmosphere containing 5% CO_2. They were left undisturbed for 3 days after which the medium was exchanged. Ten days after the start of the culture, the explants were removed and

culture medium was exchanged again. Cells were detached with 0.083% trypsin-EDTA when numerous colonies of adherent cells were observed.

6.3.3. Proliferation studies

Undifferentiated MSC isolated from UCB, PB or UCM were observed during 5 passages and within each harvesting, cell concentration was determined in order to calculate the cell-doubling number (CDN) and the population-doubling time (PDT) using following formula: CDN= ln (N_f/N_i) / ln 2, where N_f is the final number of cells and N_i the initial number of cells, and PDT= cell culture time (in days) / CDN.

6.3.4. Tri-lineage differentiation

After two passages, approximately 1×10^6 undifferentiated MSC were used to perform the tri-lineage differentiation experiments, as previously described (De Schauwer, 2011). Non-induced cells in expansion medium were used as negative controls.

Osteogenic differentiation was performed in six-well culture dishes with approximately 3000 undifferentiated MSC/cm^2 which were cultured in expansion medium until 90-100% confluency was reached. Subsequently, osteogenic differentiation was induced with osteogenic medium which was exchanged twice weekly, and evaluated after 20 days of culture using the Alizarine Red S histological staining as well as by detecting alkaline phosphatase activity (Millipore®, Overijse, Belgium).

Chondrogenic differentiation was performed using a micromass culture system, i.e. 2.5×10^6 cells were centrifuged in 15-mL Falcon tubes at 150 ×g for 5 min at RT after which the chondrogenic medium was added without disturbing the cell pellet. The medium was

exchanged every 3-4 days during three weeks after which the chondrogenic differentiation was evaluated by the Alcian blue histological staining.

To initiate the adipogenic differentiation, 2.1×10^4 undifferentiated MSC/cm^2 were seeded in six-well culture dishes and cultured until 100% confluency. Subsequently, cells were exposed to four cycles of 72h culturing in the adipogenic induction medium and 24h of culturing in the adipogenic maintenance medium, followed by five consecutive days of culturing in adipogenic maintenance medium. The Oil Red O histological staining was used to detect the intracellular accumulation of lipid droplets.

6.3.5. Media

The UCB and PB culture medium contained low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 30% fetal calf serum (FCS) (GIBCO), 10^{-7} M low dexamethazone, 50 µg/mL gentamycine, 10 µl/mL antibiotic antimycotic solution, 250 ng/mL fungizone (all from Sigma) and 2 mM ultraglutamine (Invitrogen), based on the medium described by Koch et al. (2007). The expansion medium was identical to the culture medium but without dexamethasone. The UCM culture medium contained low-glucose DMEM (Invitrogen), 15% FCS (GIBCO), 50 µg/mL gentamycine, 10 µl/mL antibiotic antimycotic solution, and 250 ng/mL fungizone (all from Sigma).

For the tri-lineage differentiation experiments, following media were used: (i) osteogenic medium, containing low glucose DMEM (Invitrogen), 10% FCS (GIBCO), 0.2 mM L-ascorbic acid-2-phosphate (Fluka), 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 µg/mL gentamycine and 10 µl/mL antibiotic antimycotic solution (all from Sigma); (ii) chondrogenic medium based on the basal differentiation medium (Lonza), complemented with 10 ng/mL Transforming Growth Factor β3 (Sigma) and (iii) adipogenic induction medium containing DMEM-LG (Invitrogen), 1 µM dexamethasone, 0.5 mM 3-

isobutyl-1-methylxanthine, 10 µg/mL rh-insuline, 0.2 mM indomethacin, 15% rabbit serum, 50 µg/mL gentamycine and 10 µl/mL antibiotic antimycotic solution (all from Sigma); (iv) adipogenic maintenance medium which was identical to the adipogenic induction medium except for the omission of dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine.

6.3.6. Immunophenotypical profile

Undifferentiated equine MSC from the third or fourth passage were immunophenotyped using multicolor flow cytometry, as previously described (De Schauwer et al., 2012). A detailed description of monoclonal antibody (mAb) clones and dilutions are given in Table 1. Following combinations of marker panels were assessed: CD29-Alexa488/MHC II-RPE/CD44-APC/7-AAD (subset 1), CD105-RPE/CD90-Alexa647/7-AAD (subset 2), CD45-Alexa488/CD73-RPE/7-AAD (subset 3), and the monocyte marker-Alexa488/CD79α-Alexa647 (subset 4). To identify the viable cells, 7-AAD was used in the first three subsets. The presence of MHC I on the cell surface of the undifferentiated MSC was analyzed separately as this mAb could not be included in one of the subsets.

In the subsets without cell permeabilization, approximately 2×10^5 cells per tube were centrifuged to pellet in DMEM + 1% bovine serum albumin (BSA) and incubated for 15 min at 4°C in the dark with following non-labeled primary mAbs: MHC II (subset 1), CD90 (subset 2), and CD73 (subset 3), respectively. After two washing steps, cells were incubated with a secondary Ab conjugated with a relevant fluorochrome for 15 min at 4°C in the dark (Table 1). To remove the excess of secondary Ab, cell pellets were washed twice after which a 15-min blocking step using 10% mouse serum was performed to exclude non-specific binding of the directly labeled primary mAbs on the secondary Ab. Subsequently, these directly labeled primary mAbs i.e. CD29 and CD44 (subset 1), CD105 (subset 2), and CD45 (subset 3), respectively, were incubated for 15 min at 4°C in the dark. After three washing

steps, cell pellets were finally resuspended in 400µl PBS and analyzed after 10 min incubation with 7-AAD for all three subsets of markers.

The MSC which were used to detect the presence of MHC I on the cell surface, followed the same protocol, i.e. after centrifugation, cells were incubated for 15 min at 4°C in the dark with the non-labeled MHC I mAb, washed twice and next, incubated with the RPE-conjugated secondary Ab for 15 min at 4°C in the dark. After three washing steps, the cell pellet was resuspended in 400µl PBS and analyzed.

For the intracellular antigen detection in subset 4, cells were fixed and permeabilized using Fix and Perm® (Caltag, Invitrogen, Gent, Belgium), according to the manufacturer's instructions. Next, a blocking step was provided using 10% horse serum during 15 min in the dark at RT, after which the CD79α and the monocyte marker primary mAbs were incubated for 15 min at 4°C in the dark. The pellet was resuspended in 400µl PBS after three washing steps.

For all tubes, at least 10,000 cells were analyzed using a FACSCanto flow cytometer (Becton Dickinson Immunocytometry systems) equipped with two lasers, a 488 nm solid state and a 633 nm HeNe laser, and FACSDiva software. All data were compensated and corrected for autofluorescence as well as for unspecific bindings using both secondary Ab and/or isotype negative controls. Compensation for spectral overlap between fluorochromes was performed using an automatic calibration technique (FACSDiva software, Becton Dickinson) and subsequently evaluated individually with a matrix.

Table 1. Overview of the marker panels of primary mAbs and 7-AAD to immunophenotype viable equine MSC using multicolor flow cytometry. Relevant isotype controls and secondary antibodies for indirectly labeled markers are also provided.

	subset	Marker	Company	Clone	Sec Ab	Dilution
Multicolor FCM	1	CD29-Alexa488^{+}	Biolegend	TS2/16		1:20
		MHC II^{-}	Serotec	CVS20	RPE	1:50
		7-AAD^{-}	Calbiochem			
		CD44-APC^{+}	Becton Dickinson	IM7		1:20
	2	CD105-RPE^{+}	Serotec	SN6		1:10
		7-AAD^{-}	Calbiochem			
		CD90^{+}	VMRD	DH24A	Alexa647	1:100
	3	CD45-Alexa488^{-}	Serotec	F10-89-4		1:5
		CD73^{+}	Abcam	10f1	RPE	1:5
		7-AAD^{-}	Calbiochem			
	4	Monocyte-Alexa488^{-}	Serotec	MAC387		1:2.5
		CD79α-Alexa647^{-}	Serotec	HM57		1:2.5
Single color FCM		MHC I^{+}	VMRD	PT85A	RPE	1:66
Secondary Ab	1 & 3	Sheep anti-mouse IgG-RPE	Sigma			1:20
	2	Goat anti-mouse IgG-Alexa647	Invitrogen			1:200
Isotype controls	1&4	Mouse IgG1-Alexa488	Biolegend			1:20
	1&2&3	Mouse IgG1-RPE	Biolegend			1:10
	1	Rat IgG2b-APC	Biolegend			1:20
	2	Mouse IgM	Becton Dickinson		Alexa647	1:50
	3	Mouse IgG2a-Alexa488	Biolegend			1:20
	4	Mouse IgG1-Alexa647	Biolegend			1:100

6.3.7. Gating strategy

The population of interest (P1) was visualized on the area versus width signal of the forward scatter (FSC-A/FSC-W) dot plot, after which a second gate was placed on the area versus width signal of the side scatter (SSC-A/SSC-W) dot plot (P2), as such discriminating for doublets and clumps. Next, the single cell population was identified by defining P2 on a side scatter area signal versus a forward scatter area (SSC-A/FSC-A) signal dot plot. Finally,

a Boolean gate was placed on the single cell population and the 7-AAD^{neg} cells to enable the analysis of the viable single cell population.

6.3.8. Statistical analysis

Data were presented as mean ± standard deviation. Linear regression models were fit to determine the association between the source of MSC (UCB, PB, UCM) and the moment of first observation, the moment of first passage, cell proliferation data, i.e. CDN and PDT, and the expression of markers related to cell immunogenicity, i.e. MHC I and MHC II. In all models, mare was forced into the model to correct for clustering. To approximate normality, a reciprocal (CD105) or arcsin-transformation (CD44, CD90, subset 2, CD79, MHC I, monocyte marker, subset 4) of the different markers was performed. A reciprocal transformation was also used to obtain a normal distribution of the PDT. Statistical significance was assessed at $P < 0.05$. The fit of the models was evaluated by examination of the normal probability plots of residuals and by inspection of the residuals plotted against the predicted values. Least square means were calculated. All analyses were performed using SPSS 19.0 (SPSS, Inc. Headquarters).

6.4. Results

6.4.1. Success rate of isolating equine MSC

Umbilical cord blood, PB and UCM were collected from 6 mares with a normal parturition which delivered healthy, viable foals. No complications were encountered for both mares and foals upon sampling. Putative equine MSC could be isolated from all 3 sources. However, due to late contamination of 4/6 UCM cultures (at 17.8 ± 4.8 days), only in 2/6 UCM samples, MSC could be observed. For the UCB and PB samples, a success rate of isolation of 100% was noted (Table 2). Interestingly, adherent cells could be observed in the UCB samples on average as early as 8 days post culturing, in contrast to PB and UCM samples, where adherent cells were first spotted around 14 days (Table 2). Adherent cell

populations derived from either UCB, or PB or UCM, displayed the same spindle-shaped fibroblast-like cells morphology (Fig. 1 A-C).

Table 2. Descriptive statistics of the success rate ± standard deviation (SD, %), moment of first observation ± SD (days), and moment of first passage ± SD (days) of the isolation of putative equine MSC from 6 umbilical cord blood (UCB), peripheral blood (PB) and umbilical cord matrix (UCM) samples, respectively.

Source	Success rate (%)	First observation (days)	First passage (days)
UCB	100	8.5 ± 2.7	15.8 ± 2.1
PB	100	14.5 ± 3.6	19.7 ± 2.9
UCM	33.3*	14.5 ± 0.7	21.5 ± 4.9

*Contamination in the UCM cultures occurred at 17.8 ± 4.8 days, i.e. long after expected MSC growth.

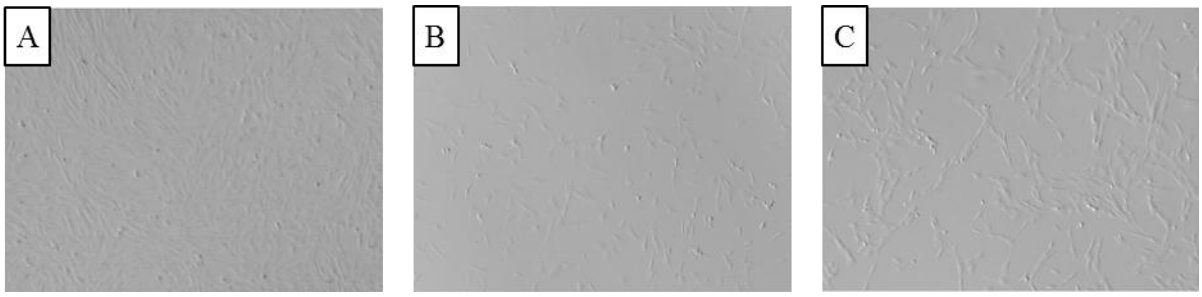

Figure 1. Adherent spindle-shaped cells derived from UCB (A,40x), PB (B,40x) and UCM (C,40x). Cells from all 3 sources demonstrated similar cell morphologies.

6.4.2. Cell proliferation

The obtained values for both CDN and PDT, without taking the initial lag phase into account, indicated that MSC from all three sources were able to rapidly divide *in vitro* (Fig. 2). Concerning the CDN, no statistically significant differences were observed between sources (Fig. 2A). Moreover, the PDT did not substantially differ between the different sources although on passage 3, the PDT of UCB-derived MSC (1.24 ± 0.28 days) was

significantly lower than the PDT of PB- and UCM-derived MSC (1.75 ± 0.61 days and 1.64 ± 0.69 days, respectively) and this was shown to be primarily due to the source-specific cell division patterns (Table 3). The PDT of UCB increased from 1.20 ± 0.26 at passage 2 up to 2.23 ± 1.31 at passage 5 while the PDT of PB and UCM, respectively, steeply increased from 1.24 ± 0.27 and 1.10 ± 0.25 at passage 2 to 1.75 ± 0.61 and 1.64 ± 0.69 at passage 3 and then subsequently decreased to 1.71 ± 0.58 and 1.08 ± 0.24 at passage 4 and to 1.29 ± 0.31 and 1.13 ± 0.27 at passage 5, respectively (Fig. 2B).

Table 3. Linear regression model describing the effect of the source of equine mesenchymal stromal cells (MSC) and the passage on the reciprocal transformed population doubling time (PDT).

Independent variable	n[1]	Estimate[2]	SE[3]	LSM[4]	P-value
Mare	6	…	…	…	< 0.001
Source					0.12
Umbilical cord blood	6	ref.	…	1.49	…
Peripheral blood	6	0.02	0.05	1.47	0.75
Umbilical cord matrix	2	0.16	0.08	1.21	0.04
Passage					0.03
2	14	ref.	…	1.18	…
3	12	-0.18	0.07	1.51	0.01
4	11	-0.15	0.07	1.42	0.03
5	10	-0.14	0.07	1.42	0.04
Source x passage[5]	…	…	…	…	< 0.001

[1] Number of observations
[2] Reciprocal transformation of population doubling time
[3] Standard error of the mean
[4] Back-transformed least square means
[5] Estimates are not shown. See Figure 2 for least square mean values for different comparisons

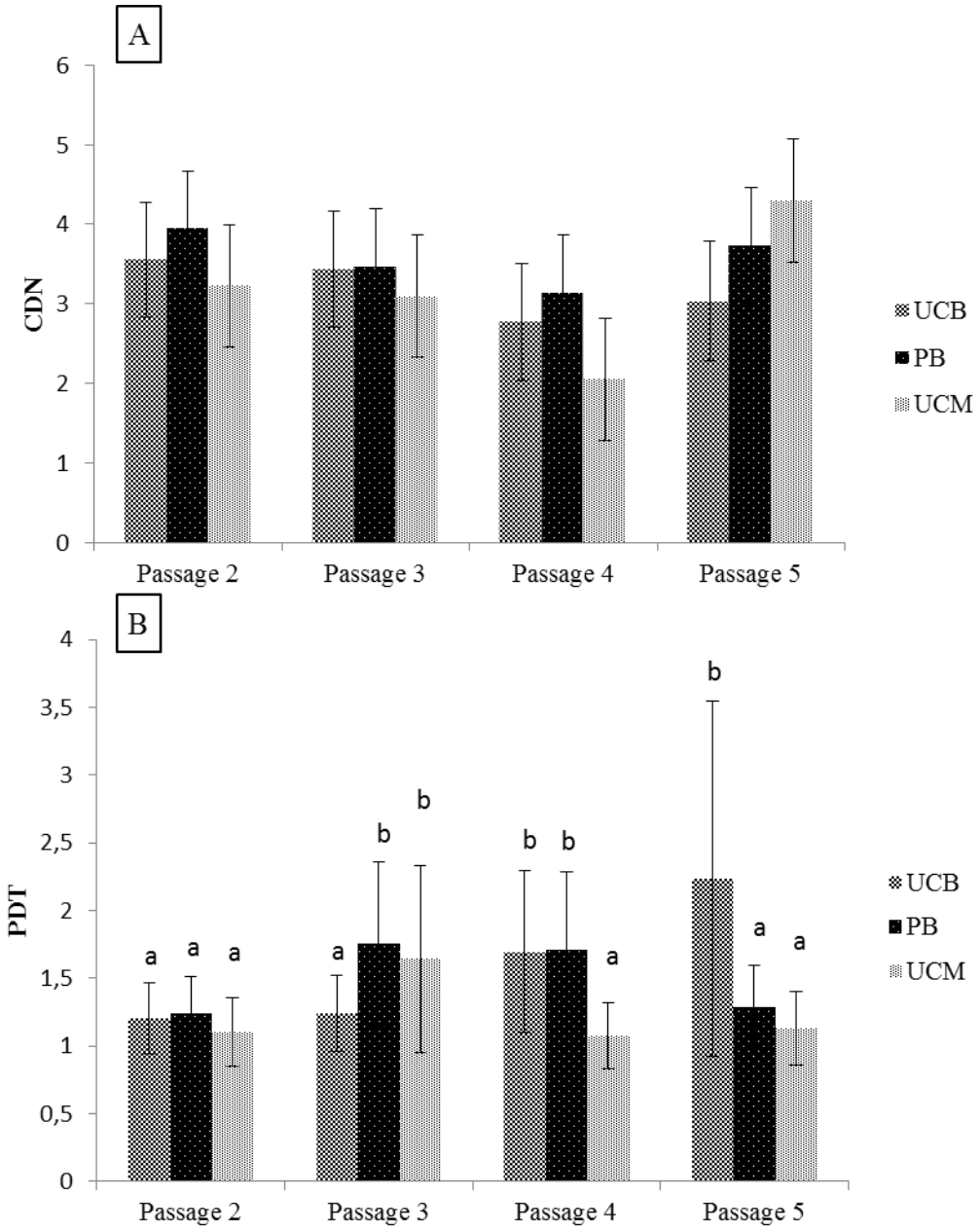

Figure 2. Proliferation data of mesenchymal stromal cells (MSC) derived from umbilical cord blood (UCB), peripheral blood (PB) and umbilical cord matrix (UCM) for passage 2 to 5, expressed by the least square means ± standard deviation of the cell doubling number (CDN) (**A**) and the back-transformed least square means of the population doubling time (PDT) (**B**). Different superscripts (a,b) denote statistically significant differences between either sources or passages not sharing the same superscript.

6.4.3. Tri-lineage differentiation potential

Osteogenic differentiation was confirmed by an increased expression of alkaline phosphatase activity and the Alizarine Red S histological staining which identifies the osteogenic specific calcium deposits (Taylor & Clegg, 2011). It was found that equine UCB-derived MSC were able to differentiate into osteocytes (Fig. 3A & B) although it needs to be mentioned that in 2 out of the 6 samples, the negative control group was also slightly positive for the alkaline phosphatase activity (Fig. 3C) whereas in one other sample, no calcium deposits could be detected with Alizarine Red S (Fig. 3D). Osteogenic differentiation could also be confirmed for the PB-derived MSC (Fig. 3E & F) although it was also here noted that in 2 out of the 6 samples only a slight difference in alkaline phosphatase activity was seen between the negative control and the differentiated group. In one other PB sample, the Alizarine Red S staining was negative and the alkaline phosphatase activity positive. This PB sample originated from the same mare for which the UCB sample was Alizarine Red S negative. In MSC isolated from the 2 non-contaminated UCM samples, the increased alkaline phosphatase activity could be clearly demonstrated while none of the samples was positive for the Alizarine Red S staining (Fig. 3G & H).

No difference in chondrogenic differentiation potential was noticed for MSC derived from either UCB, PB or UCM as all cells differentiated towards the chondrogenic lineage were clearly positive for the Alcian blue histological staining which identifies the proteoglycans present in the chondrogenic matrix (Taylor & Clegg, 2011) (Fig. 3I).

Also for the adipogenic differentiation potential, the Oil Red O staining demonstrated the intracellular accumulation of lipid droplets in differentiated MSC from all three sources (Fig. 3J).

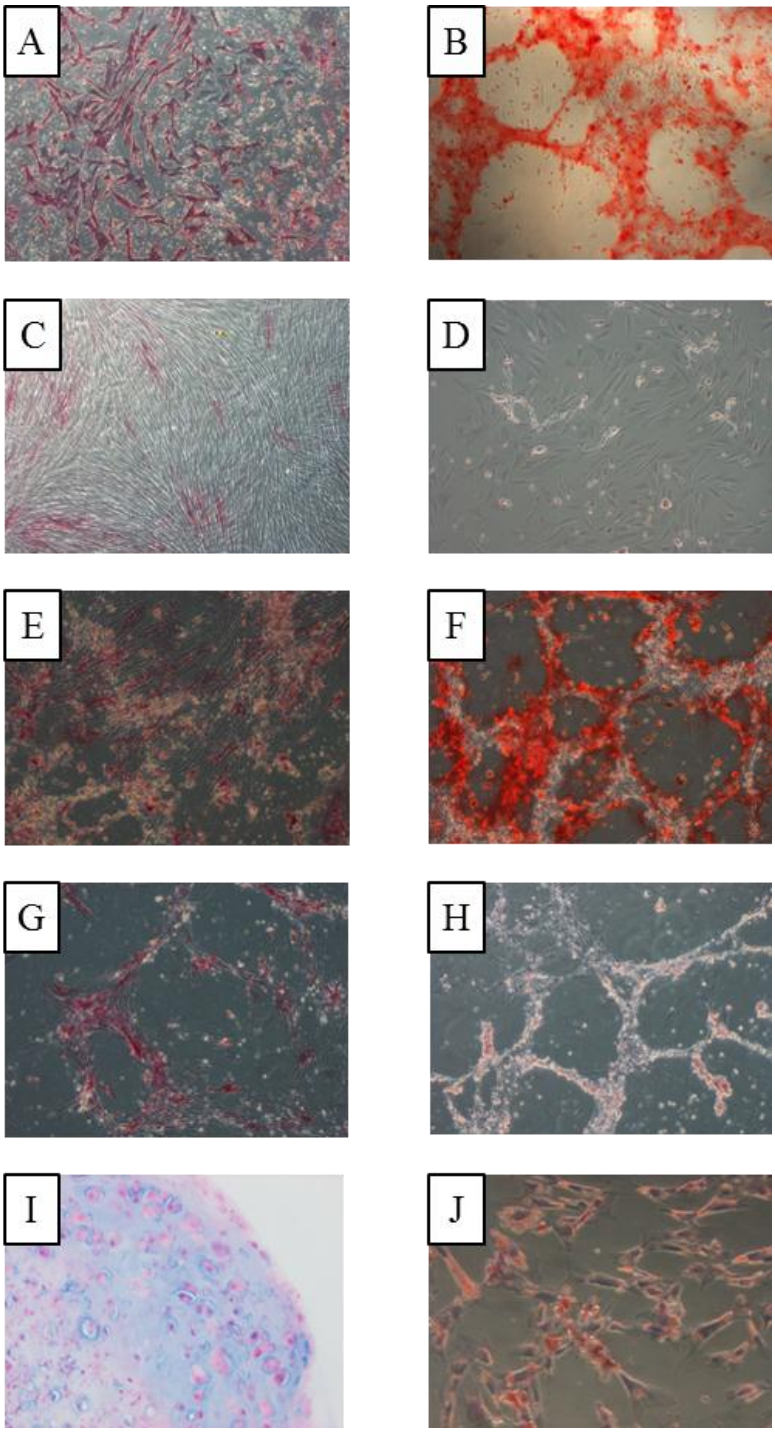

Figure 3. Tri-lineage differentiation potential. Isolated equine UCB-derived MSC were able to differentiate towards the osteogenic lineage, as demonstrated by an increased alkaline phosphatase activity (A,10x) and the Alizarine Red S staining (B,10x), although in 2 out of 6 samples, the negative control group was slightly positive for alkaline phosphatase activity (C,10x) and one other sample was negative for Alizarine Red S (D,10x). MSC isolated from PB are also capable of osteogenic differentiation, as confirmed by alkaline phosphatase activity and Alizarine Red S (E & F, respectively,10x). MSC derived from UCM are staining positive for alkaline phosphatase activity (G,10x) but negative for Alizarine Red S (H,10x). A representative example was shown to demonstrate the chondrogenic and adipogenic differentiation (I,40x & J,20x, respectively).

6.4.4. Immunophenotypical profile

An overview of the results obtained for both the single and the combined expression of the different markers from the four subsets on equine MSC derived from either UCB or PB or UCM is given in Table 4. On average 90.0 ± 8.8 % and 90.0 ± 0.7 % of the UCB- and UCM-derived MSC, respectively, simultaneously fulfilled the expression criteria of subset 1 ($CD29^{pos}$, MHC II^{neg}, 7-AAD^{neg}, $CD44^{pos}$), while this was only the case for 82.8 ± 5.4 % of the PB-derived MSC. The expression of CD105 (subset 2) included 1 outlier on the UCM-derived MSC which was 91.7% positive, resulting in an average expression of 21.9 ± 30.1% of the UCM-derived MSC. In contrast, UCB- and UCM-derived MSC were 2.6 ± 3.4 % and 1.0 ± 1.4 % CD105 positive, respectively. The percentage of viable MSC in subset 3 ($CD45^{neg}$ and $CD73^{pos}$) was identical for all three sources, i.e. 0.3 ± 0.4 % for UCB-derived MSC and 0.3 ± 0.1 % for PB- and UCM-derived MSC. In subset 4, the difference in lack of expression of the monocyte marker and CD79α was significant albeit very small, i.e. 99.7 ± 0.2 % for UCB-derived MSC, 97.9 ± 1.3 % for PB-derived MSC, and 99.0 ± 1.2 % for UCM-derived MSC. The expression of MHC I varied between sources and ranged from 62.7 ± 24.8 % for PB-derived MSC to 80.3 ± 12.9 % for UCB-derived MSC.

6.5. Discussion

Three minimal-invasive sources for equine MSC, i.e. UCB, PB and UCM, were compared regarding their most important characteristics. These include success rate of isolation, proliferation potential, differentiation capacities and immunophenotypical profile. To minimalize animal-dependent influences between the different sources, samples were matched by comparing all tissue sources within the same horse. As such, the large variability in age and breed of the horses was significantly reduced (Ahern et al., 2011). Based on the

data obtained in the current study, we propose UCB as the most valuable, non-invasive alternative to bone marrow-derived equine MSC.

Table 4. Immunophenotypical characterization of equine MSC derived from UCB, PB and UCM, expressed as the percentage (%) of cells either positive or negative for each of the 10 markers. Data are presented as average ± standard deviation (n=6, except for UCM, n=2). Subset marker combination results are shown in bold.

Marker	UCB	PB	UCM	P-value
CD29	99.6 ± 0.2	98.2 ± 0.9	98.4 ± 1.0	< 0.001
MHC II	99.5 ± 0.5	99.2 ± 0.7	99.2 ± 0.6	0.192
7-AAD	90.7 ± 8.9	85.5 ± 5.2	94.8 ± 0.6	< 0.001
CD44	99.2 ± 0.2	96.6 ± 4.1	97.3 ± 1.1	0.003
Subset 1	**90 ± 8.8**	**82.8 ± 5.4**	**90.9 ± 0.7**	**< 0.001**
CD105	2.7 ± 3.4	1.2 ± 1.6	46.2 ± 64.3	0.699
7-AAD	92.5 ± 7.2	86.2 ± 6.5	93.6 ± 4.2	< 0.001
CD90	99.4 ± 0.3	97.8 ± 2.4	66.9 ± 27.6	< 0.001
Subset 2	**2.6 ± 3.4**	**1.0 ± 1.4**	**21.9 ± 30.1**	**0.016**
CD45	99.5 ± 0.3	98.2 ± 0.9	98.9 ± 1.4	0.338
CD73	0.5 ± 0.5	0.8 ± 0.5	0.5 ± 0.1	0.052
7-AAD	93.8 ± 5.3	88.5 ± 5.1	95.5 ± 1.3	< 0.001
Subset 3	**0.3 ± 0.4**	**0.3 ± 0.1**	**0.3 ± 0.1**	**0.286**
Monocytes	99.8 ± 0.1	98.7 ± 1.2	99.0 ± 1.3	0.017
CD79	99.9 ± 0.1	99.0 ± 1.0	99.9 ± 0	< 0.001
Subset 4	**99.7 ± 0.2**	**97.9 ± 1.3**	**99.0 ± 1.2**	**0.001**
MHC I	80.3 ± 12.9	62.7 ± 24.8	77.6 ± 11.8	< 0.001

Obtaining contamination-free UCM cultures was found to be problematic, making this tissue a less appealing source for equine MSC. Fungal or bacterial contamination has also been described in other studies using UCM as a source for MSC (Passeri et al., 2009; Lovati et al., 2011; Iacono et al., 2012). A potential explanation for the high contamination rate in our UCM samples could be that these samples were processed after the umbilical cord had ruptured spontaneously. Since horses are foaling in a non-sterile environment, contact with feces and the mare's perineum is inevitable. To decrease the risk of contamination, the umbilical cord could alternatively be tied at both ends with cable ties and separated from the

foal and the placental tissue before it ruptures spontaneously (Bartholomew et al., 2009; Toupadakis et al., 2010; Carrade et al., 2011; Lovati et al., 2011). In the present study, the umbilical cord was nevertheless processed after it ruptured spontaneously in order not to interfere with the physiological process of parturition. Indeed, when either the mare or the foal moves, the umbilical cord of a foal usually ruptures at a natural stricture, being 2-3 cm from the foal's ventral abdominal wall, resulting in a constriction of the cord vessels and practically no blood loss (Christensen, 2010).

In marked contrast, no contamination problems were observed when culturing either UCB or PB. Moreover, in our hands, an isolation success rate of 100% was found for both sources, while varying percentages have been described by others. Koch et al. (2007) were the first to report the isolation of equine MSC from UCB with a success rate of 57%. Since then, isolation percentages ranging from 75% to 100% were reported by the latter group and others (Koch et al., 2009; Shuh et al., 2009; De Schauwer et al., 2011; Iacono et al., 2012). For PB, equine MSC were initially isolated in 36.4% of the obtained blood samples (Koerner et al., 2006) and this percentage increased until 44% and 66.6% in later studies (Giovannini et al., 2008; Martinello et al., 2010). The isolation success rate of 100% in the present study indicates that isolation methods and culture conditions have been optimized in recent years. Interestingly, we found that the appearance of adherent cell colonies was approximately twice as fast for UCB samples compared to PB samples, i.e. 8.5 versus 14.5 days respectively. This could not be explained by the number of cells originally seeded, as this was kept constant for all samples tested. It might, however, be explained by the fact that the concentration of circulating MSC in PB is likely very low (Koerner et al., 2006; Martinello et al., 2010; Ahern et al., 2011; Dhar et al., 2012). In human medicine, apheresis is used to obtain higher numbers of MNC from PB since this automatic centrifugation technique allows to process large volumes of PB, as such obtaining a homogenous MNC harvest with minimal RBC

contamination. So far, only one study was done to obtain equine MSC via apheresis, based on the hypothesis that a large number of MSC would be isolated after culturing the isolated MNC (Aher et al., 2011). However, although the MNC and platelets were concentrated and RBC contamination was reduced, the MNC were not able to adhere to plastic culture flasks nor were they capable of tri-lineage differentiation (Ahern et al., 2011). As the explants of the UCM were immediately transferred to culture flasks and cells were supposed to migrate from these explants, it is not known how many UCM-derived cells were initially seeded.

Also in our proliferation studies, the UCB-derived MSC showed to be superior compared to those of the other 2 sources tested. Although the data on CDN and PDT suggest that equine MSC from all three sources could be easily expanded, it was found that the PDT for UCB-derived MSC gradually increased while the PDT was initially higher in comparison to UCB-derived MSC but then substantially decreased for PB- and UCM-derived MSC. Consequently, substantially more UCB-derived MSC can be cryopreserved after only one passage which has important implications considering their potential future therapeutical use.

When further characterizing the equine MSC obtained from the three different sources, some interesting observations were made. Both UCB- and PB-derived MSC were capable of trilineage differentiation which is consistent with other studies (Koch et al., 2007; Giovannini et al., 2008; Shuh et al., 2009; De Schauwer et al., 2011; Dhar et al., 2012; Spaas et al., 2012). However, we were unable to confirm differentiation of UCM-derived MSC towards the osteogenic lineage as only the alkaline phosphatase activity was positive in these cells while the detection of calcium deposits was clearly lacking. The latter observation implies that UCM-derived MSC do not produce a mineralized matrix, being a critical feature of more mature stage of osteogenesis, and thus do not differentiate towards osteocytes. Our findings are in contrast with previous studies which also demonstrated osteogenic differentiation based on Alizarine Red and/or Von Kossa histological staining (Hoynowski et al., 2007; Passeri et

al., 2009; Corradetti et al., 2011; Iacono et al., 2012). However, in a study of Toupadakis et al. (2010), it was demonstrated that increasing the serum concentration in the osteogenic culture medium from 10% to 20%, enhanced the osteogenesis of the UCM-derived MSC. The differentiation medium in the present study contained only 10% serum which might explain why no mineralized matrix was observed in the UCM-derived MSC induced to osteogenic differentiation. Nevertheless, these are rather preliminary conclusions as equine MSC were only isolated in 2 UCM samples.

Different sources of equine MSC were compared to assess their capacities to be ultimately used, either in an autologous or allogeneic way, in clinical cell-based therapies. Therefore, expression of the markers MHC I and MHC II which are related to cell immunogenicity, were additionally evaluated. Equine MSC from all sources showed a moderate to high expression of MHC I, ranging from 62.7 ± 24.8% for PB to 80.3 ± 12.9% for UCB, while the expression of MHC II was lacking. To our knowledge, the expression of MHC I on equine PB-derived MSC has not yet been described, but for both UCB as UCM, our results were in concordance with previous data in horses (Carrade et al., 2011; Corradetti et al., 2011; Lovati et al., 2011). Only in one study of Hoynowski et al. (2007), a negative expression of MHC I for UCM-derived MSC was observed. Unexpectedly, significant differences were observed between the expression levels of the markers upon immunophenotypical profiling the equine MSC from the three sources. The question should be asked, however, if these differences are of any relevance since both the absolute difference between the three sources and the variation within each source are small. Such differences in marker expression might well just represent the biological diversity among the animals, as suggested previously by Pascucci et al. (2011). Nevertheless, for all three subsets, the percentage of viable PB-derived MSC was less than 90% which is in marked contrast with the viability data demonstrated for the UCB- and UCM-derived MSC.

6.6. Conclusion

In the present study, a comparative analysis was carried out with equine UCB-, PB- and UCM-derived MSC originating from the same horse. Due to high contamination risks and low success rates in isolating MSC, UCM seemed less interesting as alternative source for equine MSC compared to bone marrow or adipose tissue. Especially UCB and, to a lesser extent, PB were shown promising alternatives for the current more invasive sources for equine MSC. It took nearly twice as long in comparison to UCB samples to isolate MSC from all PB samples and as flow cytometrically demonstrated, the percentage of viable PB-derived MSC was significantly lower. Nevertheless, UCB- and PB-derived MSC shared both other determined characteristics, e.g. differentiation capacities and immunophenotypical profile. We suggest that future research in this rapidly evolving field should focus on comparative studies on the immunogenicity of MSC according to their source to determine whether or not source differences exist which lead to the identification of a preferred source for therapeutical applications of equine MSC.

6.7. Acknowledgements

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References

Ahern BJ, Schaer TP, Terkhorn SP, Jackson KV, Mason NJ, Hankenson KD. Evaluation of equine peripheral blood apheresis product, bone marrow, and adipose tissue as sources of mesenchymal stem cells and their differentiation potential. Am J Vet Res 2011;72:127-133.

Bartholomew S, Owens SD, Ferraro GL, Carrade DD, Lara DJ, Librach FA, Borjesson DL, Galuppo LD. Collection of equine cord blood and placental tissues in 40 Thoroughbred mares. Eq Vet J 2009;41(8):724-728.

Berg L, Koch T, Heerkens T, Bessonov K, Thomsen P, Betts D. Chondrogenic potential of mesenchymal stromal cells derived from equine bone marrow and umbilical cord blood. Vet Comp Orthop Traumatol 2009;22(5):363-370.

Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004;22:625-634.

Borjesson DL, Peroni JF. The regenerative medicine laboratory: facilitating stem cell therapy for equine disease. Clin Lab Med 2011;31(1):109-123.

Braun J, Hack A, Weis-Klemm M, Conrad S, Treml S, Kohler K, Walliser U, Skutella T, Aicher WK. Evaluation of the osteogenic and chondrogenic differentiation capacities of equine adipose tissue-derived mesenchymal stem cells. Am J Vet Res 2010;71:1228-1236.

Brehm W, Burk J, Delling U, Gittel C, Ribitsch I. Stem cell-based tissue engineering in veterinary orthopaedics. Cell Tissue Res 2012;347:677-688.

Carrade DD, Affolter VK, Outerbridge CA, Walson JL, Galuppo LD, Buerchler S, Kumar V, Walker NJ, Borjesson DL. Intradermal injections of equine allogeneic umbilical cord-derived mesenchymal stem cells are well tolerated and do not elicit immediate or delayed hypersensitivity reactions. Cytotherapy 2011(a);13(10):1180-1192.

Christensen BW. Parturition. In: McKinnon AO, Squires EL, Vaala WE, Varner DD. Equine Reproduction. West Sussex, Wiley-Blackwell, second edition, 2011, pp. 2268-2276.

Corradetti B, Lange-Consiglio A, Barucca M, Cremonesi F, Bizzaro D. Size-sieved subpopulations of mesenchymal stem cells from intervascular and perivascular equine umbilical cord matrix. Cell Prolif 2011;44:330-342.

De Schauwer C, Meyer E, Cornillie P, De Vliegher S, Van de Walle GR, Hoogewijs M, Declercq H, Govaere J, Demeyere K, Cornelissen M, Van Soom A. Optimization of the isolation, culture and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng Part C 2011;17(11):1061-1070.

De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JLJ, Braeckmans K, Van Soom A, Meyer E. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry part A 2012;81A(4):312-323.

Dhar M, Neilsen N, Beatty K, Eaker S, Adair H, Geiser D. Equine peripheral blood-derived mesenchymal stem cells: isolation, identification, trilineage differentiation and effect of hyperbaric oxygen treatment. Eq Vet J 2012;doi: 10.111/j.2042-3306.2011.00536.x.

Giovannini S, Brehm W, Mainil-Varlet P, Nesic D. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. Differentiation 2008;76:118-129.

Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T, Mitchell KE. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem Biophys Res Commun 2007;362:347-353.

Iacono E, Brunori L, Pirrone A, Pagliaro PP, Ricci F, Tazzari PL, Merlo B. Isolation, characterization and differentiation of mesenchymal stem cells from amniotic fluid, umbilical cord blood and Wharton's jelly in the horse. Reproduction 2012;143:455-468.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

Koch TG, Thomsen PD, Betts DH. Improved isolation protocol for equine cord blood-derived mesenchymal stromal cells. Cytotherapy 2009;11:443-447.

Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP. Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. Stem Cells 2006;24:1613-1619.

Lange-Consiglio A, Corradetti B, Bizzaro D, Magatti M, Ressel L, Tassan S, Parolini O, Cremonesi F. Characterization and potential applications of progenitor-like cells isolated from horse amniotic membrane. J Tissue Eng Regen Med 2011;doi:10.1002/term.565.

Lovati AB, Corradetti B, Lange-Consiglio A, Recordati C, Bonacina E, Bizzaro D, Cremonesi F. Comparison of equine bone marrow-, umbilical cord matrix and amniotic fluid-derived progenitor cells. Vet Res Commun 2011;35:103-121.

Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M. Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood. Tissue Eng Part C 2010;16:771-781.

Mensing N, Gasse H, Hambruch N, Haeger JD, Pfarrer C, Staszyk C. Isolation and characterization of multipotent mesenchymal stromal cells from the gingiva and the periodontal ligament of the horse. BMC Vet Res 2011;7:42.

Nixon AJ, Dahlgren LA, Haupt JL, Yeager AE, Ward DL. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. Am J Vet Res 2008;69(7):928-937.

Pascucci L, Gurina G, Mercati F, Marini C, Dall'Aglio C, Paternesi B, Ceccarelli P. Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: towards the definition of minimal stemness criteria. Vet Immunol Immunopathol 2011;144:499-506.

Passeri S, Nocchi F, Lamanna R, Lapi S, Miragliotta V, Giannessi E, Abramo F, Stornelli MR, Matarazzo M, Plenteda D, Urciuoli P, Scatena F, Coli A. Isolation and expansion of equine umbilical cord-derived matrix cells. Cell Biol Int 2009;33:100-105.

Shuh EM, Friedman MS, Carrade DD, Li J, Heeke D, Oyserman SM, Galuppo LD, Lara DJ, Walker NJ, Ferraro GL, Owens SD, Borjesson DL. Identification of variables that optimize isolation and culture of multipotent mesenchymal stem cells from equine umbilical cord blood. Am J Vet Res 2009;70:1526-1534.

Smith RKW, Korda M, Blunn GW, Goodship AE. Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. Equine Vet J 2003;35:99-102.

Spaas JH, De Schauwer C, Cornillie P, Meyer E, Van Soom A, Van de Walle GR. An *in vitro* characterization of equine peripheral blood mesenchymal stromal cells. The Vet J 2012, in press.

Stewart AA, Barrett JG, Byron CR, Yates AC, Durgam SS, Evans RB, Stewart MC. Comparison of equine tendon-, muscle-, and bone marrow-derived cells cultured on tendon matrix. Am J Vet Res 2009;70(6):750-757.

Taylor SE, Clegg PD. Collection and propagation methods for mesenchymal stromal cells. Vet Clin North Am Equine Pract 2011;27(2):263-274.

Toupadakis CA, Wong A, Genetos DC, Cheung WK, Borjesson DL, Ferraro GL, Galuppo LD, Leach JK, Owens SD, Yellowley CE. Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res 2010;71(10):1237-1245.

Vidal MA, Walker NJ, Napoli E, Borjesson DL. Evaluation of senescence in mesenchymal stem cells isolated from equine bone marrow, adipose tissue, and umbilical cord tissue. Stem Cells Dev 2012;21:273-283.

CHAPTER 7

GENERAL DISCUSSION

When this PhD research was drafted in 2006, little was known about equine mesenchymal stem cells (MSC). Moreover, since there was no previous experience in our laboratory with stem cells and long-term cell culture, all procedures had to be optimized in order to establish standard operating procedures. Mainly literature from human medicine was initially used to unravel how to isolate equine MSC. Soon after our first trial, several studies were published on MSC in horses with a varying degree of characterization of these cells. Remarkably, little or no standardized criteria were available to describe the characteristics of equine MSC in sharp contrast to the detailed guidelines available for the unequivocal characterization of human MSC. As stated in 2006, human MSC must be able to (i) adhere to plastic, (ii) differentiate towards the osteogenic, chondrogenic and adipogenic lineage, and (iii) display a panel of selected mesenchymal markers while lacking known hematopoietic markers (Dominici et al., 2006).

The general aim of this thesis was to optimize the characterization protocols for equine MSC following the guidelines described for human MSC. Plastic-adherency is a common feature of both human and equine MSC which can be used to separate the MSC from the hematopoietic non-adherent cells at the start of the culture, as the latter are removed when changing the culture medium (Taylor et al., 2007). However, the protocols described for both differentiating and immunophenotyping human MSC required further optimization before being applicable for equine MSC. Therefore, we will focus in this final chapter on the results obtained in this research for (1) the collection and isolation, (2) the culture and tri-lineage differentiation and (3) the immunophenotyping of equine MSC. Based on these findings, we would like to propose guidelines applicable for equine MSC.

Collection and isolation of equine MSC

A successful MSC isolation starts with a proper collection of the sample. Obviously, the collection of UCB requires a supervised parturition. Hereby, working sterile is emphasized as contamination was initially one of the major problems when isolating and culturing MSC. The non-sterile environment where the mare gives birth and the possible contact with the mare's perineal area are complicating factors when sampling UCB. Usually, the hind legs of the foal stay in the genital tract of the mare immediately after parturition. As illustrated in Figure 1, the umbilical cord is clamped as closely as possible to the vulva in order not to interfere with normal parturition and as such, allowing the umbilical cord to rupture at the natural stricture (indicated by the arrow). Otherwise, there is a substantial risk that the cord vessels are not constricted and a considerable blood loss occurs through the umbilicus. Moreover, the foal will be more susceptible to infections of the umbilicus with the actual risk of complications such as septicemia and polyarthritis, eventually possibly leading to the death of the foal. To prevent these complications in the comparative study (Chapter 6), the umbilical cord was processed after it ruptured spontaneously although this decision implied an increased risk of contamination of the umbilical cord matrix (UCM) cultures. After all, the well-being of the foal remains primordial.

After clamping, the umbilical cord is disinfected with 70% alcohol before the umbilical vessel is punctured and UCB is drained by gravity into a sterile standard 350-mL blood donor bag containing CPD A anticoagulant (Terumo®), and subsequently stored at 4°C. Adapting the critical conditions described for human UCB (Bieback et al., 2004), equine UCB samples were only processed if (1) the total volume exceeded 150 mL, as such avoiding that the UCB was too much diluted by the anticoagulant, (2) storage time was less than 15 hours, and (3) there were no signs of coagulation or hemolysis.

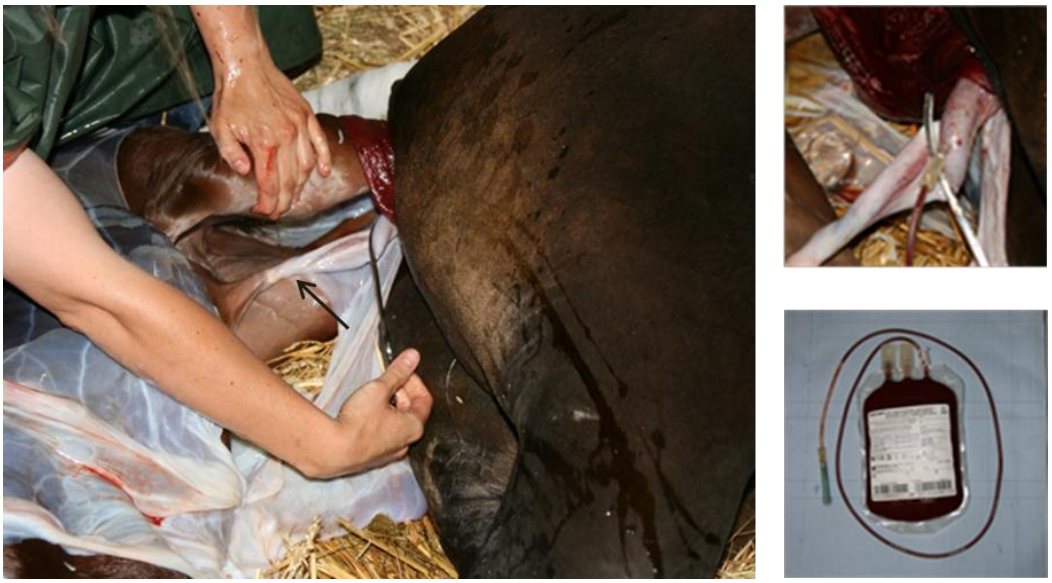

Figure 1. Collection of UCB. Immediately after parturition, the umbilical cord is clamped as closely as possible by the vulva of the mare and disinfected with 70% alcohol. The natural stricture where the umbilical cord usually ruptures is indicated by the arrow. After puncturing the umbilical vessel, as shown in the upper right panel, the UCB is drained by gravity into a sterile blood donor bag (lower right panel).

After sample collection optimization, the next crucial step for obtaining equine MSC is a successful isolation technique. In humans, several UCB fractionation procedures for the isolation of human MSC have been proposed based on the partial or complete removal of RBC and plasma (Regidor et al., 1999) and based on this information, the major aim of our first study was to compare four isolation methods to acquire equine MSC from UCB (Chapter 3). Hereby, we found that equine MSC could be obtained successfully when isolating the mononuclear cells (MNC) using density gradient separation methods. More specifically, it was found that Percoll gave a significantly better yield in comparison to Ficoll-Paque. In contrast, HES and NH_4Cl proved unsuccessful for the isolation of equine UCB-derived MSC. Ficoll-Paque is the most commonly used density medium to isolate MSC while this study clearly showed that Percoll gave a significantly better yield. Nevertheless, the 2 isolation protocols were not completely comparable as the buffy coat fraction was used in the Percoll

protocol while whole blood was used in the Ficoll-Paque protocol. Moreover, other volumes were used, i.e. 7 mL diluted buffy coat suspension was layered on 7 mL Percoll in the first protocol while 30 mL undiluted UCB was loaded on 10 mL Ficoll-Paque in the other protocol, which is technically more challenging. Therefore, it might be recommended to compare both density gradient methods by loading the same volume of the same cell suspension on the respective density gradients. As such, it can be unambiguously investigated whether Percoll compares favourably to Ficoll-Paque.

Banking of equine UCB-derived MSC

After processing the UCB, isolated MNC can either be directly cultured to derive MSC for storage in an UCB-MSC bank or be cryopreserved and stored to isolate MSC later on for either research and/or clinical applications. Although it has been previously reported that it is difficult to isolate and expand equine MSC from cryopreserved UCB (Koch et al., 2007), we demonstrated that equine MSC can be successfully cultured from cryopreserved UCB-derived MNC samples (Chapter 5). This strategy avoids the use of a time-consuming work-up protocol requiring both equipment and experienced personnel to start the cultures immediately upon collection (Casado-Diaz et al., 2008). This aspect of the procedure is mainly interesting from a commercial point of view as the downside is that MSC still have to be expanded upon thawing the cryopreserved MNC at the moment of injury. As such, these MSC are no longer readily available and the time for cellular expansion must also be taken into account, hereby losing the most important advantage of UCB-derived MSC when compared to bone marrow-derived MSC.

Our freezing solution consists of high-glucose DMEM, 10% FCS and 20% dimethylsulfoxide (DMSO), as described by Koch et al. (2007), in which both the FCS and

the DMSO should be replaced or at least reduced when these MSC are intended to use in clinical therapies. When freezing cells, cryoprotective agents are indispensable in the medium to better control the ice crystal formation and cell damage that occurs due to the intra- and extracellular accumulation of these crystals. Although the commonly used DMSO is considered as relatively non-toxic, a variety of side-effects may occur following reinfusion of cryopreserved cells with DMSO. This toxicity might be related to the concentration of DMSO which is used in the freezing solution, but can also result from cell lysis material (Liseth et al., 2005). Most of the excessive DMSO can be removed by washing the cryopreserved cells after thawing. Nevertheless, it is desirable to reduce the amount of DMSO in cryopreserved cells without jeopardizing the quality of the stem cell product (Liseth et al., 2005). Therefore, protocols for cryopreserving equine MSC should be further optimized covering critical parameters such as choice and concentration of cryoprotectans, and cryopreservation techniques (vitrification versus slow freezing-rapid thawing).

Optimization of the culture and tri-lineage differentiation of equine MSC

Optimization of undifferentiated equine MSC culturing conditions

The possible clinical applications of MSC in cell therapy for horses, as described for humans, requires the refinement of culture media as well as well-defined culture conditions (Mannello & Tonti, 2007). We showed that culture parameters such as seeding density, FCS-coating or common stem cell media, did not have a significant influence on the success rate of equine MSC isolation (Chapter 3). Two different culture media were compared for the isolation and expansion of MSC from UCB. MesenCult® is a commercial MSC medium based on McCoy's medium while the other culture medium was home-made and largely based on the medium described by Koch et al. (2007). Culturing putative MSC was successful using

either of both media. Therefore, the home-made culture medium was used in subsequent experiments. Nevertheless, it must be mentioned that the latter medium contains 30% FCS. Such a high percentage is commonly supplied to culture media as growth supplement because of its high levels of growth stimulatory factors, i.e. plasma proteins to promote cellular adhesion and endogenous growth factors to stimulate proliferation, and its low levels of growth inhibitory factors (Mannello & Tonti, 2007; Toupadakis et al., 2010). As demonstrated in the study of Toupadakis et al. (2010), equine UCB-derived MSC did not proliferate in medium containing only 10% serum while sufficient expansion occurred when using medium containing 20% serum. Nevertheless, the use of FCS for expansion of cells which might possibly be used *in vivo*, is controversial. Because FCS is chemically ill-defined, a high degree of serum variability within and between suppliers and batches occurs (Dimarakis & Levicar, 2006). Besides, there is a potential risk that bovine serum proteins can be internalized in stem cells and as such, stimulate immunogenicity (Mannello & Tonti, 2007). More specifically, immunological reactions could arise due to the presence of bovine proteins attached to the cultured cells that can become antigens after transplantation (Dimarakis & Levicar, 2006). Furthermore, the possible contamination of the FCS with prions, viruses, and zoonotic agents, must always be kept in mind (Berger et al., 2006). On the other hand, several clinical trials using human MSC cultured in FCS containing medium, were unable to demonstrate any significant side effects (Berger et al., 2006; Sotiropoulou et al., 2006). Moreover, it has been proven that FCS contains bioactive factors which decrease the cytotoxic consequences induced by necrotic and apoptotic signals (Mannello & Tonti, 2007). For example, human MSC cultured in the presence of FCS were more efficient in suppressing the alloantigen-induced lymphocyte proliferation, suggesting that FCS-containing medium might be more suitable when applying allogeneic MSC (Mannello & Tonti, 2007). Autologous serum might be a valuable alternative in order to eliminate or reduce the risk of

above-mentioned side effects (Berger et al., 2006). However, most types of cellular therapy require large numbers of MSC, which means large amounts of culture media and subsequently, large volumes of peripheral blood to obtain autologous serum (Sotiropoulou et al., 2006). Therefore, it is probably not appropriate for future 'off-the-shelf' availability of stem cell therapy although it is a safe and reliable solution for small clinical studies (Dimarakis & Levicar, 2006). Another possibility to obtain a culture setting that is free from animal-derived products and as such more defined, is using plant hydrolysates as a serum substitute (T'Joen et al., 2012). These are often added in microbial culture media to stimulate the microbial, fungal and bacterial growth, in industrial fermentation processes as well as in vaccine production (T'Joen et al., 2012). A recent study comparing hydrolysates of different origin and in different concentrations, demonstrated that Hypep 4601, a product originating from wheat, appears to be the most efficient substitute for FCS in human embryonic stem cell cultures (T'Joen et al., 2012).

Considering following arguments: (1) the achievement of more stringent control levels, (2) the avoidance of any prion, viral, or zoonose contamination risks, (3) the reduction of possible immunological reactions, and (4) the decrease in the demand on animal supplies, it can be concluded that in preclinical settings, all animal-derived products should ideally be excluded and synthetic recombinant alternatives should be used instead as substitutes (Dimarakis & Levicar, 2006). For example, a defined, xeno-free and serum-free version of the commercial medium which was initially used in our experiments, was recently launched on the market, i.e. MesenCult®-XF. As there was no difference between both media in our hands for isolating MSC, it is recommended to test the serum-free version of the MesenCult® for future application of equine MSC in clinical cell-based therapies.

Optimization of the tri-lineage differentiation of equine MSC

Culture media are not only of utmost importance for proper culturing of equine MSC, but highly defined media are also of paramount importance for differentiating MSC to the osteogenic, chondrogenic and adipogenic cell lines *in vitro*. In this thesis, 33 MSC samples were succesfully initiated towards these three lineages although preliminary experiments were found to be essential to optimize the differentiation conditions and to carefully interpret the histological stainings.

As stated by the International Society for Cellular Therapy (ISCT), osteogenic differentiation can be demonstrated using the Alizarine Red S or Von Kossa histological staining to identify mineral deposition (Dominici et al., 2006). The expression and organization of these matrix proteins are essential prerequisites for proper tissue function and engineered tissue constructs (Arnhold et al., 2007). In our first study (Chapter 3), osteogenic differentiation was confirmed by increased alkaline phosphatase activity and both these histological stainings. Alkaline phosphatase is regarded as one of the important enzymes taking part in mineralization processes (Laczka-Osyczka et al., 1998). Although it is not a minimal requirement as defined by the ISCT, demonstrating an increased alkaline phosphatase activity is the most frequently used marker to confirm osteogenic differentiation (Alves et al., 2011). However, in both chapters 3 and 6, undifferentiated MSC of some negative control groups showed a weak positivity for alkaline phosphatase although no mineralization could be demonstrated. These findings are also described in literature as this parameter of early osteoblast differentiation is not unique to osteogenic differentiated cells (Declercq et al., 2005). For instance, it has been shown in murine embryonic stem cell cultures that a decreasing alkaline phosphatase activity is one of the earliest indicators of differentiation induction (O'Connor et al., 2008). This implies that an increased alkaline phosphatase activity can also be considered as a stemness marker (Martinello et al., 2010). In

conclusion, the formation of a mineralized extracellular matrix is the most reliable parameter for final osteoblast differentiation (Declercq et al., 2005). Calcium deposition can be confirmed using Alizarine Red S while phosphate salts are identified with the Von Kossa staining (Taylor & Clegg, 2011). Still, it should be mentioned that the silver ions used in the Von Kossa staining can react with phosphate and as such, cause false-positive areas of black staining (Taylor & Clegg, 2011).

Initially, chondrogenic differentiation experiments failed when using a home-made differentiation medium. After switching to a commercial basal differentiation medium (Lonza), the chondrogenic differentiation was confirmed macroscopically using a micromass culture system and histologically by Alcian blue staining (Chapter 3). Performing the histological staining on the negative control group, however, turned out to be less straightforward as no matrix was formed keeping the cells together in the micromass culture system. Therefore, MSC in adherent cultures without chondrogenic differentiation medium were used as negative controls.

Concerning the adipogenic differentiation, Janderova et al. (2003) demonstrated that replacing FCS with rabbit serum improved the adipogenic differentiation of human MSC. In the study of Koch et al. (2007), different protocols, either with or without rabbit serum, were used to induce adipogenic differentiation in equine MSC. Only when rabbit serum was added to the medium, adipogenic differentiation could be verified (Koch et al., 2007). Rabbit serum contains a high concentration of free fatty acids which play an important role during the first days of the differentiation process from pre-adipose to adipose cells (Giovannini et al., 2008). Moreover, polyunsaturated fatty acids are transactivators of PPAR-γ, a transcription factor which expression increases during the adipogenic differentiation process as it is implicated in adipogenesis (Giovannini et al., 2008). However, during the differentiation period of 21 days, many differentiated cells detached and were lost when replacing the medium. The rabbit

serum has been identified as responsible factor for this observation (Burk, personal communication). Therefore, it would be advisable to shorten the differentiation period as preliminary experiments showed that the intracellular accumulation of lipid droplets in differentiated MSC can already be identified after two cycles of adipogenic differentiation.

Optimization of the immunophenotypical characterization of equine MSC

For human MSC, it has been defined that these cells must express CD73, CD90, and CD105 and lack expression of CD14, CD34, CD45, CD79α and MHC II (Dominici et al., 2006). The lack of a single marker specific for MSC and the currently limited availability of monoclonal antibodies (mAbs) recognizing equine epitopes, are major complicating factors for the immunophenotypical characterization of equine MSC. Consequently, we searched for cross-reactivity by single color flow cytometry (Chapter 4) investigating whether or not mAbs directed against human, murine and canine epitopes, recognize the equine epitopes using appropriate control groups. Concurrent cytospin slides were prepared to confirm the flow cytometrical findings. As such, we were able to list a subset of mAbs which can now be applied by other equine MSC research groups to independently challenge the reliability of our results. Indeed, contradicting results on the cross-reactivity of mAbs with equine epitopes, are reported. As such, we were unable to confirm the cross-reactivity of any of the five tested clones for CD34 on equine endothelial cells although three of these clones were recently used in other studies to characterize equine MSC (Hoynowski et al., 2007; Martinello et al., 2010; Marfe et al., 2012).

As stated by the ISCT, immunophenotyping of MSC is preferably performed by multicolor flow cytometry to simultaneously demonstrate the co-expression of specific MSC markers and the absence of hematopoietic antigen expression (Dominici et al., 2006; Xie et

al., 2010). Flow cytometry is a highly sensitive and specific method for the qualitative and quantitative assessment of multiple parameters of individual cells in suspension (Reggeti & Bienzle, 2011). However, it is susceptible to false-positive signals resulting from the autofluorescence of cells, lack of antibody titration, nonspecific antibody binding, interactions of different fluorochromes, lack of instrument optimization, etc. (Radcliffe et al., 2010; Reggeti & Bienzle, 2011). Therefore, it is important to ensure the quality instrument performance, use appropriate and sufficient controls, and verify that the detected signals indeed correspond to a specific antigen-antibody interaction (Reggeti & Bienzle, 2011). As such, examining cytospin slides by fluorescence microscopy is a common confirmatory method (Reggeti & Bienzle, 2011). As described in our standard operating flow cytometrical procedure (Chapter 4), antibodies were titrated and three types of negative controls were included: cells without antibody (to check for autofluorescence), cells with only fluorochrome-linked secondary antibody and appropriate isotype controls. Especially in subset 4 where cytoplasmatic antigens were identified, the latter negative controls were indispensable since antibody binding to non-specific targets and dead cells increases after fixation and permeabilization (Reggeti & Bienzle, 2011). Furthermore, when identifying epitopes on the cell membrane using indirect stainings (as in subsets 1-3), mouse serum was used to block non-specific bindings since both secondary antibodies used were anti-mouse. In subset 4, a blocking step with horse serum was used to minimize the non-specific bindings. Finally, the antigen-antibody interaction was confirmed by cytospin slides of the cells which were obtained from the same samples screened with flow cytometry.

Viable equine MSC simultaneously expressed CD29 and CD44, and lacked expression of MHC II. They also simultaneously lacked expression of CD79α and the monocyte marker. Due to the more variable expression of CD73 and CD105, less straightforward results were obtained for the combined expression of both these markers. Nevertheless, equine MSC

clearly express CD90 and lack expression of CD45. Our obtained results for CD73 were recently confirmed by Pascucci et al. (2011) and this raises the intriguing question whether the criteria as defined for human MSC can be applied for equine MSC as well. Concerning the expression of pluripotency markers, species-specific differences have been reported, including differences in morphology, patterns of antigen immunostaining, expression of differentiation markers, as well as expression profiles of cytokines, cell cycle, and cell death-regulating genes (Ginis et al., 2004). Regarding the adult stem cell markers, one canine study corroborates our results by reporting that MSC from dogs also lack expression of both CD73 and CD105 (Vieira et al., 2010), again suggesting that the expression of these markers might be species-dependent. Still, other influences such as the MSC source or the cell detaching agent used can contribute to the observed differences between studies. It has indeed been recently demonstrated that detaching equine cells with trypsin can damage certain cell surface proteins like CD14 while other markers such as CD90 appeared unaffected (Hackett et al., 2011). Further research is therefore indicated to identify which equine epitopes are trypsin-labile.

Future prospects

With the research on equine MSC still being in its infancy, many questions remain to be answered. When focusing on the aspect of characterization of equine MSC, guidelines as described for human MSC have been as good as accomplished: plastic-adherency and tri-lineage differentiation has been unambiguously demonstrated and the detailed immunophenotypical profile of equine MSC has now been provided in this PhD research. However, it is not yet unequivocally clear whether equine MSC differ from human MSC with respect to other critical aspects. Thus, to be confident about the unique features of the equine

MSC immunophenotype, the absence of CD34 expression should be demonstrated on the protein level and the varying results obtained for CD73 and CD105 must be further clarified.

The ultimate goal of most fundamental research is the translation towards the clinical practice and for equine MSC in particular, the implementation in cell-based therapies. As aforementioned, all animal-derived products should ideally be excluded and synthetic recombinant alternatives should be used instead (Dimarakis & Levicar, 2006). Xeno-free and serum-free commercial media should be evaluated for culturing equine MSC in order to replace the undefined FCS and to obtain a completely defined culture medium. As these media are often very expensive and their exact composition is proprietary knowledge and thus unknown, the aforementioned use of plant hydrolysates should be explored as well. Excluding animal-derived products should also be applied in the freezing solutions used to cryopreserve equine MSC.

Considering possible future clinical applications, it has been suggested that some healing properties of MSC are influenced by tissue source, implying that the choice of MSC source might play a role depending on the kind of injury for which a treatment is desired (Borjesson & Peroni, 2011). It is most likely that allogeneic equine MSC will increasingly be used in cell-based therapies. It is known that MSC derived from neonatal sources such as UCB and UCM exhibit immunosuppressive properties (Carrade et al., 2011a) and are therefore preferable allogeneic MSC than those isolated from more adult sources such as bone marrow or PB. Such comparative studies on immunogenicity of MSC according to source have not been performed yet in human or in veterinary medicine. The onset of these studies has been performed in this doctoralthesis by evaluating the presence and absence of MHC I and MHC II, respectively. In horses, only one study has been performed so far concerning the immunogenic properties of MSC in which it was determined if allogeneic equine UCM-derived MSC were antigenic using standard intradermal testing *in vivo* and mixed lymphocyte

reactions *in vitro* (Carrade et al., 2011b). This study demonstrated that allogeneic equine MSC did not elicit an acute graft rejection when injected intradermally or a delayed-type hypersensitivity response when injected intradermally after previous sensitization (Carrade et al., 2011b).

Last but not least, it should be highlighted that in human medicine, many additional steps beyond characterization *in vitro* must be undertaken before a cell-based therapy reaches the patients. After optimizing the protocols for isolation, expansion, and differentiation, the safety as well as the quality of the cell products must be extensively studied. Subsequently, the product potency is evaluated in pre-clinical studies where toxicity, working dose, efficacy and possible adverse effects are determined. Finally, after performing clinical trials ranging from phase I to phase IV, the cell product can be approved and finalized to be launched on the market (T'Joen et al., 2012) (Fig. 2). In veterinary medicine, on the contrary, stem cell therapies are not rigorously supervised by regulatory agencies (Koch et al., 2009). So, cell-based therapies are implemented in veterinary medicine without demonstrating the efficacy *in vitro* or in preclinical animal studies (Fortier & Travis, 2011). Therefore, future research must also aim at optimizing these therapies by focusing on source influence, isolation, enrichment and processing procedures of MSC, as well as on the timing, route of administration, formulation, and dosing of such cell-based therapies (Fortier & Travis, 2011). As such, blind randomized control trials must be designed in order to unambiguously demonstrate the efficacy of cell-based therapies in equine medicine (Clegg & Pinchbeck, 2011).

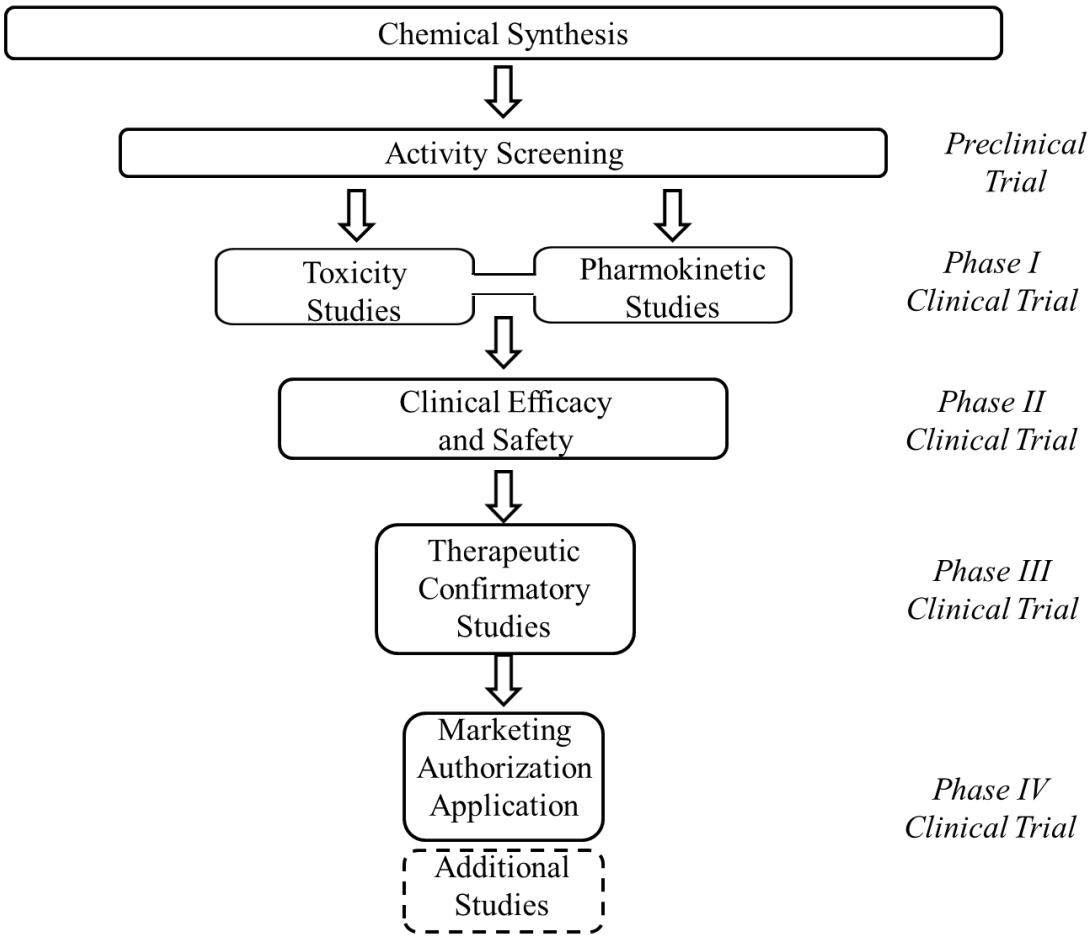

Figure 2. Extrapolation of experimental data to human medicine. Potential therapeutic products are tested in preclinical trials to identify potential target organs as well as parameters for clinical monitoring, to confirm the absence of unacceptable side-effects and to estimate the initial safe starting dose. In phase I trials, the tolerance and pharmakinetic features of the product are evaluated. Subsequently, the efficacy and safety are assessed: both on a small number of patients in Phase II trials as on a large scale in patient populations (Phase III trials). Phase IV trials include post-approval studies delineating additional information such as the drugs' risks and optimal use. Many products do not reach the end of the clinical trials as indicated by the decreasing width of the boxes.

Concluding remarks

Based on the results obtained in this thesis, following conclusions can be drawn:

1. although Ficoll-Paque is the most commonly used method to isolate equine MSC, Percoll was found to be the preferable gradient medium to process equine umbilical cord blood in order to obtain high numbers of equine MSC.

2. our goal of the in-depth characterization of equine MSC based on both the tri-lineage differentiation and their detailed immunophenotypical profile has been accomplished as we identified cross-reacting mAbs which allowed us to demonstrate the presence of CD29, CD44, and CD90 on the cell surface of equine MSC, and the absence of CD45, CD79α, a monocyte marker and MHC II. Our optimized multicolor flowcytometry protocol could therefore be promoted to immunophenotype equine MSC.
3. when comparing three minimal-invasive sources of equine MSC, UCB and, to a lesser extent, PB are valuable alternatives for bone marrow or adipose tissue. Due to the high contamination risks and an unsuccessful differentiation towards the osteogenic lineage, UCM appears to be a less suitable source of equine MSC.
4. the successful isolation of equine MSC from the cryopreserved mononuclear cells was demonstrated. This opens up novel opportunities since mononuclear cells from equine UCB samples can be cost- and time-efficiently banked for future autologous or allogeneic therapeutic use.

References

Alves H, Dechering K, Van Blitterswijk C, De Boer J. High-throughput assay for the identification of compounds regulating osteogenic differentiation of human mesenchymal stromal cells. Plos One 2011;6(10):e26678.

Arnhold SJ, Goletz I, Klein H, Stumpf G, Beluche LA, Rohde C, Addicks K, Litzke LF. Isolation and characterization of bone marrow-derived equine mesenchymal stem cells. Am J Vet Res 2007;68:1095-1105.

Berger MG, Veyrat-Masson R, Rapatel C, Descamps S, Chassagne J, Boiret-Dupre N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cell 2006;24:2888-2890.

Bieback K, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004;22:625-634.

Borjesson DL, Peroni JF. The regenerative medicine laboratory: facilitating stem cell therapy for equine disease. Clin Lab Med 2011;31(1):109-123.

Carrade DD, Affolter VK, Outerbridge CA, Walson JL, Galuppo LD, Buerchler S, Kumar V, Walker NJ, Borjesson DL. Intradermal injections of equine allogeneic umbilical cord-derived mesenchymal stem cells are well tolerated and do not elicit immediate or delayed hypersensitivity reactions. Cytotherapy 2011(a);13(10):1180-1192.

Carrade DD, Owens SD, Galuppo LD, Vidal MA, Ferraro GL, Librach F, Buerchler S, Friedman MS, Walker NJ, Borjesson DL. Clinicopathologic findings following intra-articular injection of autologous and allogeneic placentally derived equine mesenchymal stem cells in horses. Cytotherapy 2011(b);13(4):419-430.

Casado-Diaz A, Santiago-Mora R, Jimenez R, Caballero-Villarraso J, Herrera C, Torres A, Dorado G, Quesada-Gomez JM. Cryopreserved human bone marrow mononuclear cells as a source of mesenchymal stromal cells: application in osteoporosis research. Cytotherapy 2008;10:460-468.

Clegg PD, Pinchbeck GL. Evidence-based medicine and stem cell therapy: how do we know such technologies are safe and efficacious? Vet Clin North Am Equine Pract 2011;27(2):373-382.

Dimarakis I, Levicar N. Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cells 2006;24:1407-1408.

Declercq HA, Verbeek RMH, De Ridder LIFJM, Schacht EH, Cornelissen MJ. Calcification as an indicator of osteoinductive capacity of biomaterials in osteoblastic cell cultures. Biomaterials 2005; 26:4964-4974.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.

Fortier LA, Travis AJ. Stem cells in veterinary medicine. Stem Cell Research & Therapy 2011;2:9.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, Amit M, Hoke A, Carpenter MK, Itskovitz-Eldor J, Rao M. Differences between human and mouse embryonic stem cells. Dev Biol 2004;269:360-380.

Giovannini S, Brehm W, Mainil-Varlet P, Nesic D. Multilineage differentiation potential of equine blood-derived fibroblast-like cells. Differentiation 2008;76:118-129.

Hackett CH, Flaminio MJBF, Fortier LA. Analysis of CD14 expression levels in putative mesenchymal progenitor cells isolated from equine bone marrow. Stem Cells Dev 2011;20:721-735.

Hoynowski SM, Fry MM, Gardner BM, Leming MT, Tucker JR, Black L, Sand T, Mitchell KE. Characterization and differentiation of equine umbilical cord-derived matrix cells. Biochem Biophys Res Commun 2007;362:347-353.

Janderova L, McNeil M, Murrell AN, Mynatt RL, Smith SR. Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes Res 2003;11(1):65-74.

Koch TG, Heerkens T, Thomsen PD, Betts DH. Isolation of mesenchymal stem cells from equine umbilical cord blood. BMC Biotechnol 2007;7:26.

Koch TG, Berg LC, Betts DH. Current and future regenerative medicine – principles, concepts, and therapeutic use of stem cell therapy and tissue engineering in equine medicine. Can Vet J 2009;50:155-165.

Laczka-Osyczka A, Laczka M, Kasugai S, Ohya K. Behavior of bone marrow cells cultured on three different coatings of gel-derived bioactive glass-ceramics at early stages of cell differentiation. J Biomed Mater Res 1998;42:433-442.

Liseth K, Abrahamsen JF, Bjørsvik S, Grøttebø K, Bruserud Ø. The viability of cryopreserved PBPC depends on the DMSO concentration and the concentration of nucleated cells in the graft. Cytotherapy 2005;7:328-333.

Mannello F, Tonti G. Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement, nonconditioned medium, or ad hoc formula? All that glitters is not gold! Stem Cells 2007;25:1603-1609.

Marfe G, Massaro-Giordano M, Ranalli M, Cozzoli E, Di Stefano C, Malafoglia V, Polettini M, Gambacurta A. Blood derived stem cells: an ameliorative therapy in veterinary ophthalmology. J Cell Physiol 2012;227(3):1250-1256.

Martinello T, Bronzini I, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M. Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood. Tissue Eng Part C 2010;16:771-781.

O'Connor MD, Kardel MD, Iosfina I, Youssef D, Lu M, Li MM, Vercauteren S, Nagy A, Eaves CJ. Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human ambryonic cells. Stem Cells 2008;26:1109-1116.

Pascucci L, Gurina G, Mercati F, Marini C, Dall'Aglio C, Paternesi B, Ceccarelli P. Flow cytometric characterization of culture expanded multipotent mesenchymal stromal cells (MSCs) from horse adipose tissue: towards the definition of minimal stemness criteria. Vet Immunol Immunopathol 2011;144:499-506.

Radcliffe CH, Flaminio MJBF, Fortier LA. Temporal analysis of equine bone marrow aspirate during establishment of putative mesenchymal progenitor cell populations. Stem Cells Dev 2010;19:269-281.

Reggeti F, Bienzle D. Flow cytometry in veterinary oncology. Vet Pathol 2011;48(1):223-235.

Regidor C, Posada M, Monteagudo D, Garaulet C, Somolinos N, Forés R, Briz M, Fernandez MN. Umbilical cord blood banking for unrelated transplantation: evaluation of cell separation and storage methods. Exp Hematol 1999;27:380-385.

Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Cell culture medium composition and translational adult bone marrow-derived stem cell research. Stem Cells 2006;24:1409-1410.

T'Joen V, Declercq H, Cornelissen M. Towards clinical-grade hESC culture and cryopreservation for tissue engineering purposes, PhD thesis, 2012.

Taylor SE, Clegg PD. Collection and propagation methods for mesenchymal stromal cells. Vet Clin North Am Equine Pract 2011;27(2):263-274.

Taylor SE, Smith RKW, Clegg PD. Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet J 2007;39:172-180.

Toupadakis CA, Wong A, Genetos DC, Cheung WK, Borjesson DL, Ferraro GL, Galuppo LD, Leach JK, Owens SD, Yellowley CE. Comparison of the osteogenic potential of equine mesenchymal stem cells from bone marrow, adipose tissue, umbilical cord blood, and umbilical cord tissue. Am J Vet Res 2010;71(10):1237-1245.

Vieira NM, Brandalise V, Zucconi E, Secco M, Strauss BE, Zatz M. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 2010;19:279- 289.

Xie W, Wang X, Du W, Liu W, Qin X, Huang S. Detection of molecular targets on the surface of $CD34^{+}CD38^{-}$ bone marrow cells in myelodysplastic syndromes. Cytometry part A 2010;77A:840-848.

SUMMARY

The potential clinical use of mesenchymal stromal cells (MSC) in equine veterinary medicine has been increasingly explored the past few years. Initially, little or no standardization for the isolation and characterization of equine MSC was present, in marked contrast to the detailed guidelines described for the characterization of human MSC. Therefore, the general aim of this doctoral thesis was to gain a better fundamental insight into the characteristics of equine MSC.

First, the isolation protocol for umbilical cord blood (UCB)-derived MSC was optimized (**Chapter 3**). For humans, a wide diversity of MSC isolation procedures from UCB, based on the partial or complete removal of RBC and plasma, has been described although only a few reports compared the efficacy of different isolation methods in terms of cell recovery. In veterinary medicine, two studies have been published comparing different isolation protocols for equine MSC which were derived from equine BM or from UCB, respectively. In our study, a sedimentation method using hydroxyethyl starch (HES) and a method based on the lysis of red blood cells (RBC) using ammonium chloride (NH_4Cl) were compared with two density gradient separation methods (Ficoll-Paque and Percoll). Adherent cell colonies could be established using all four isolation methods. The mononuclear cell (MNC) recovery after Percoll separation, however, resulted in significantly more putative MSC colonies while the latter were only observed on a single occasion when using HES and NH_4Cl. Culture conditions such as cell density and medium or serum coating of the wells did not significantly affect putative MSC recovery. To confirm the mesenchymal identity, isolated MSC using Percoll were subsequently differentiated towards the osteogenic, chondrogenic and adipogenic lineage, and immunophenotyped by multicolor flow cytometry based on their expression of different cell protein markers.

Since MSC markers share many common features with endothelial, epithelial and muscle cells, a panel of antigens was necessary to unequivocally identify MSC. As such, human MSC

must express CD29, CD44, CD73, CD90, and CD105 and lack expression of CD14, CD34, CD45, CD79α and MHC II. The limited availability of monoclonal antibodies (mAbs) for immunophenotyping equine cells was a major factor complicating the immunophenotypic characterization of equine MSC. Therefore, 30 commercially available monoclonal antibodies (mAbs) were validated for recognizing equine epitopes using equine MNC, equine lymphocytes or equine endothelial cells as appropriate positive control cells (**Chapter 4**). Based on the 11 cross-reacting mAb clones identified in the first part of this study, a multicolor flow cytometric protocol to immunophenotype equine MSC was developed to simultaneously demonstrate the co-expression of specific MSC markers and the absence of hematopoietic antigen expression. Equine MSC were identified as $CD29^{pos}$, MHC II^{neg}, $CD44^{pos}$, $CD45^{neg}$, $CD90^{pos}$, $CD79\alpha^{neg}$ and monocyte $marker^{neg}$. A variable expression for CD73 and CD105 on equine MSC, which is not in accordance with human MSC, was demonstrated and warrants further research including potentially critical factors such as the influence of the sources of equine MSC and the sample pretreatment.

In addition, it was investigated whether or not it is possible to cryopreserve the isolated mononuclear cell (MNC) fraction immediately after isolation and when needed, culture the MSC from the cryopreserved MNC (**Chapter 5**). This strategy avoids the use of a time-consuming work-up protocol requiring both equipment and experienced personnel to process the UCB and start the cultures upon collection. It opens up new opportunities since MNC samples from equine UCB samples can be cost- and time-efficiently banked for future research and/or possible autologous or allogeneic therapeutic use.

Traditionally, bone marrow and adipose tissue are used as equine MSC sources but issues concerning ease of isolation, expansion characteristics and donor site complications suggest the search for alternative sources. Therefore, different features of equine MSC isolated from 3 minimal-invasive sources, i.e. peripheral blood (PB), umbilical cord matrix (UCM) and UCB,

were compared and following parameters were analyzed: success rate of isolating MSC, proliferation capacity, tri-lineage differentiation ability and immunophenotypic profile (**Chapter 6**). Matched samples were obtained at parturition in order to compare the different sources within one horse as such excluding possible animal-dependent influences. Equine MSC were isolated in all the UCB and PB samples while only 2 out of 6 UCM samples yielded MSC due to contamination of the cultures. The obtained proliferation data, reflected by the cell-doubling number and the population-doubling time, indicated that equine MSC for all three sources could be easily expanded. Equine MSC from both UCB and PB could be differentiated towards the osteo- , chondro- and adipogenic lineage. Although chondrogenic and adipogenic differentiation was also confirmed for UCM-derived MSC, no mineralized matrix deposition could be detected in these cells when directed towards osteogenesis. As it is the ultimate goal of using equine MSC either autologous or allogeneic in clinical cell-based therapies, the expression of markers related to cell immunogenicity, MHC I and MHC II, was also evaluated in this thesis. Equine MSC from all sources showed a moderate to high expression of MHC I while the expression of MHC II was lacking. Significant, though probably not biologically relevant, differences in immunophenotypic expression of the remaining markers could be demonstrated. Based on the results of this study, we can conclude that UCB and, to a lesser extent, PB seem promising alternatives for bone marrow or adipose tissue. As fungal or bacterial contamination after processing the UCM is described in several studies and we were not able to confirm osteogenic differentiation of UCM-derived MSC, this source seems less interesting as a valuable alternative for equine MSC.

Based on the results obtained in this thesis, following conclusions can be drawn:

1. although Ficoll-Paque is the most commonly used method to isolate equine MSC, Percoll was found to be the preferable gradient medium to process equine umbilical cord blood in order to obtain high numbers of equine MSC.

2. our goal of the in-depth characterization of equine MSC based on both the tri-lineage differentiation and their detailed immunophenotypical profile has been accomplished as we identified cross-reacting mAbs which allowed us to demonstrate the presence of CD29, CD44, and CD90 on the cell surface of equine MSC, and the absence of CD45, CD79α, a monocyte marker and MHC II. Our optimized multicolor flowcytometry protocol could therefore be promoted to immunophenotype equine MSC.
3. when comparing three minimal-invasive sources of equine MSC, UCB and, to a lesser extent, PB are valuable alternatives for bone marrow or adipose tissue. Due to the high contamination risks and an unsuccessful differentiation towards the osteogenic lineage, UCM appears to be a less suitable source of equine MSC.
4. the successful isolation of equine MSC from the cryopreserved mononuclear cells was demonstrated. This opens up novel opportunities since mononuclear cells from equine UCB samples can be cost- and time-efficiently banked for future autologous or allogeneic therapeutic use.

In conclusion, we were the first to properly immunophenotype equine MSC. Moreover, we optimized the derivation of MSC from non-invasive sources such as UCB and PB. Our results have set the basis for UCB banking in horses, a practice which exists already for ten years in human medicine. Further research should be focused on the immunogenicity of equine MSC both *in vitro* as *in vivo*. Evidence-based clinical trials with UCB-derived MSC must be performed to confirm the therapeutical possibilities of these magical cells.

SAMENVATTING

De laatste jaren is het gebruik van mesenchymale stromale cellen (MSC) voor klinische toepassingen in de diergeneeskunde enorm toegenomen. Aanvankelijk werden de isolatie en karakterisatie van equine MSC op een weinig gestandardiseerde manier uitgevoerd, hetgeen in schril contrast stond met de gedetailleerde richtlijnen die reeds eerder beschreven waren voor de eenduidige karakterisatie van humane MSC. Daarom was de algemene doelstelling van deze doctoraatsthesis het verwerven van een beter fundamenteel inzicht in de kenmerkende eigenschappen van equine MSC.

In een eerste studie werd het protocol om MSC te isoleren uit navelstrengbloed (UCB) geoptimaliseerd (**Hoofdstuk 3**). Voor de isolatie van humane MSC uit UCB zijn verschillende isolatieprocedures beschreven die allemaal gebaseerd zijn op het gedeeltelijk of volledig verwijderen van de rode bloedcellen (RBC) en het plasma. Er zijn echter maar weinig studies uitgevoerd waarin de efficiëntie vergeleken wordt van de verschillende isolatiemethodes wat het aantal cellen betreft dat geïsoleerd kan worden. Met betrekking tot de diergeneeskunde werden er twee studies gepubliceerd: één voor equine MSC afkomstig uit beenmerg en één voor equine MSC uit UCB. In onze studie werden zowel een sedimentatiemethode met hydroxyethylzetmeel (HES) als een methode om de RBC te lyseren met ammoniumchloride (NH_4Cl) vergeleken met twee densiteitsgradiënt methodes (Ficoll-Paque en Percoll). Bij alle methodes konden adherente celkolonies worden aangetoond. Het aantal mononucleaire cellen (MNC) dat geoogst werd na Percoll isolatie, resulteerde echter in significant meer vermoedelijke MSC kolonies. Na HES en NH_4Cl isolatie werden deze kolonies maar één keer aangetoond. Andere cultuuromstandigheden zoals de concentratie waaraan de cellen geplant werden, het medium dat gebruikt werd of het al dan niet vooraf coaten met foetaal kalf serum, hadden geen significante invloed op het voorkomen van vermoedelijke MSC kolonies. Om de mesenchymale identiteit van de vermoedelijke MSC te bevestigen, werden deze na Percoll isolatie gedifferentieerd naar de osteogene, chondrogene

en adipogene cellijn. Daarnaast werden ze ook gefenotypeerd door de expressie van verschillende eiwitmarkers na te gaan met behulp van multicolor flowcytometrie.

Aangezien sommige eiwitten, die kenmerkend zijn voor MSC, ook voorkomen op endotheel-, epitheel- en spiercellen, was een panel van verschillende markers nodig om op een eenduidige manier MSC te identificeren. Zodoende dienen humane MSC de markers CD29, CD44, CD73, CD90 en CD105 tot expressie te brengen terwijl CD14, CD34, CD45, CD79α en MHC II afwezig moeten zijn. Er zijn echter maar weinig monoclonale antilichamen (mAbs) beschikbaar om equine MSC op een analoge manier te fenotyperen. Daarom werd in de volgende studie bij 30 commercieel beschikbare mAbs nagegaan of ze equine epitopen herkennen waarbij equine MNC, equine lymfocyten of equine endotheelcellen als positieve controlegroepen gebruikt werden (**Hoofdstuk 4**). Gebaseerd op de 11 kruisreagerende mAbs uit het eerste deel van de studie, werd een multicolor flowcytometrisch protocol opgesteld om equine MSC te fenotyperen waarbij zowel de aanwezigheid van bepaalde eiwitten typisch voor MSC als de afwezigheid van eiwitten kenmerkend voor hematopoietische cellen, terzelfdertijd kon worden aangetoond. Equine MSC werden geïdentificeerd als $CD29^{pos}$, MHC II^{neg}, $CD44^{pos}$, $CD45^{neg}$, $CD90^{pos}$, $CD79\alpha^{neg}$ en monocyte $marker^{neg}$. Voor CD73 en CD105 werd een variabele expressie vastgesteld, hetgeen niet in overeenstemming is met humane MSC. Toekomstig onderzoek zal verder uitwijzen of er een mogelijke invloed is van de bron waaruit de MSC gepreleveerd worden of van de producten die gebruikt worden om de adherente MSC los te maken.

Daarnaast werd ook onderzocht of het al dan niet mogelijk is om de MNC onmiddellijk na isolatie in te vriezen en later, wanneer het effectief nodig is, de MSC op te kweken uit deze ingevroren MNC (**Hoofdstuk 5**). Door deze aanpak worden de tijdrovende culturen vermeden om de MSC op te kweken uit de geïsoleerde MNC, die verder ook ervaren personeel en materiaal vereisen. Hierdoor ontstaan er nieuwe mogelijkheden voor zowel

toekomstig onderzoek als voor mogelijk autoloog of allogeen therapeutisch gebruik van MSC aangezien MNC van equine UCB stalen zowel kosten- als tijdbesparend kunnen opgeslagen worden.

Traditioneel zijn beenmerg en vetweefsel de belangrijkste bronnen om equine MSC uit te preleveren maar wegens verschillende factoren zoals het gemak waarmee MSC geïsoleerd kunnen worden, de cultuurkenmerken en mogelijke complicaties bij het preleveren, wordt gezocht naar alternatieve bronnen. Daarom werden in de laatste studie equine MSC vergeleken die verkregen werden uit drie minimaal-invasieve bronnen, met name UCB, navelstreng matrix (UCM) en perifeer bloed (PB). De volgende parameters werden daarbij vergeleken: (1) kunnen MSC geïsoleerd worden, (2) zijn er verschillen in proliferatie eigenschappen, (3) zijn de verkregen MSC in staat om te differentiëren naar osteocyten, chondrocyten en adipocyten en (4) zijn er verschillen in expressie van bepaalde markers? (**Hoofdstuk 6**). Van zes paarden werd bij de partus zowel UCB als UCM en PB verzameld waardoor de herkomst van de MSC vergeleken kon worden binnen eenzelfde dier, zodat mogelijke dierafhankelijke invloeden vermeden konden worden. Uit alle UCB en PB stalen werden MSC geïsoleerd terwijl enkel in twee van de zes UCM stalen MSC geïsoleerd konden worden. Dit laatste was een gevolg van contaminatie van een deel van de culturen. De bekomen proliferatiegegevens, die weergegeven worden door het cel verdubbelingsaantal en de populatie verdubbelingstijd, toonden aan dat MSC onafhankelijk van hun herkomst gemakkelijk konden geëxpandeerd worden. Equine MSC uit UCB en PB waren in staat om te differentiëren naar osteo-, chondro- en adipocyten terwijl die uit UCM wel differentieerden naar chondro- en adipocyten maar niet naar osteocyten, aangezien in deze groep geen gemineraliseerde osteogene matrix kon aangetoond worden. Omdat het uiteindelijke doel van equine MSC is om deze al dan niet allogeen te gebruiken in klinische toepassingen, werden in deze studie ook twee markers, MHC I en MHC II, geëvalueerd die gerelateerd zijn met

immunogeniciteit. Equine MSC afkomstig van de drie onderzochte bronnen vertoonden een matig tot hoge expressie van MHC I terwijl ze negatief waren voor MHC II. Tevens kon nog aangetoond worden dat enkele andere markers significant verschilden tussen de drie bronnen, hoewel dit hoogstwaarschijnlijk niet biologisch relevant is. Gebaseerd op de resultaten van onze studie, kunnen we besluiten dat UCB en PB veelbelovende alternatieven voor beenmerg en vetweefsel kunnen zijn. Aangezien contaminatie met schimmels en bacteriën van UCM culturen reeds beschreven is in verschillende publicaties en ook door ons als een probleem werd ervaren, en bovendien de osteogene differentiatie niet kon aangetoond worden, is UCM minder interessant als mogelijk alternatieve bron voor MSC.

Gebaseerd op de door ons verkregen onderzoeksresultaten, kunnen de volgende besluiten getrokken worden:

1. hoewel Ficoll-Paque de meest gebruikte methode is om equine MSC te isoleren, worden meer adherente MSC kolonies verkregen na Percoll isolatie waardoor deze methode te verkiezen is om UCB te verwerken teneinde MSC te isoleren.
2. de karakterisatie van equine MSC gebaseerd op enerzijds hun differentiatie vermogen en anderzijds hun fenotypisch profiel, kan gedetailleerd worden uitgevoerd. Verschillende kruisreagerende mAbs konden geïdentificeerd worden en zowel de aanwezigheid van CD29, CD44 en CD90 als de afwezigheid van CD45, CD79α, een monocyten marker en MHC II, konden aangetoond worden met behulp van flowcytometrie.
3. mesenchymale stromale cellen kunnen geïsoleerd worden uit een ingevroren MNC fractie hetgeen nieuwe mogelijkheden biedt voor autologe of allogene klinische toepassingen. Ingevroren UCB stalen kunnen zowel tijds- als kostenbesparend bewaard worden.

4. wanneer drie minimaal-invasieve herkomstbronnen van MSC vergeleken worden, zijn zowel UCB als PB veelbelovende alternatieven voor beenmerg en vetweefsel. Navelstrengmatrix kan worden beschouwd als een minder geschikte bron gezien het grote risico op contaminatie en het feit dat de osteogene differentiatie door ons niet kon worden aangetoond.

Als algemeen besluit van deze doctoraatsthesis kan gelden dat equine MSC voor het eerst volledig gefenotypeerd werden. Daarenboven werd de isolatie van equine MSC uit UCB en PB geoptimaliseerd. De verkregen resultaten hebben de basis gelegd voor het opstarten van een UCB bank voor paarden zoals die reeds een tiental jaar bij de mens bestaat. Toekomstig onderzoek moet voornamelijk gericht worden op de immunogeniciteit van equine MSC zowel *in vitro* als *in vivo*. Daarnaast moeten gecontroleerde klinische studies met equine MSC afkomstig van UCB plaatsvinden om de therapeutische mogelijkheden van MSC te onderzoeken.

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dus wens ik iedereen veel succes zowel met de praktijk als in het onderzoek! Enkele mensen wil ik toch apart vermelden. Cyriel, nu sta je nog bij de BP^+ – binnen enkele dagen ben je de nieuwe assistent paard. Ik hoop dat je evenveel van deze nieuwe uitdaging zal genieten als ik gedaan heb. Zoals je de weg naar Balegem gevonden hebt om te komen eten, hoop ik je ook regelmatig in de Westhoek te zien! Miel, ook al heb je niet veel op met paarden, dat Trojaans paard in mijn computer heb je toch kunnen bedwingen. Binnenkort is het uw beurt om hier vooraan te staan – gelukkig heb ik de weddenschap gewonnen! Ik hoop dat je je ook in West-Vlaanderen regelmatig zelf eens uitnodigt om te komen eten ☺

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Catharina

Curriculum vitae

Catharina De Schauwer werd geboren op 26 augustus 1980 te Gent. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Catharinacollege te Geraardsbergen (Latijn-Wiskunde), begon zij in 1997 met de studie Diergeneeskunde aan de Universiteit Gent. Zij behaalde haar diploma van Dierenarts in 2004 met onderscheiding, optie Herkauwers.

Op 1 oktober 2004 startte zij een internship op de dienst Buitenpraktijk van de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Op 1 oktober 2005 trad zij in dienst als voltijds assistent op diezelfde vakgroep. Haar taak bestond uit het klinische werk zowel op de Kliniek Voortplanting en Verloskunde van de Grote Huisdieren als bij klanten thuis, en het actief participeren in de nacht- en weekenddiensten. Daarnaast werd in 2006 het doctoraatsonderzoek gestart naar de isolatie van mesenchymale stamcellen uit navelstrengbloed bij paard. Tevens behaalde zij in 2012 het postgraduaatdiploma 'Vakdierenarts Paard'.

Catharina De Schauwer is auteur en co-auteur van verschillende wetenschappelijke publicaties in nationale en internationale tijdschriften en stelde haar onderzoek voor op verschillende nationale en internationale congressen.

BIBLIOGRAPHY

Publications in international peer-reviewed journals

Govaere JLJ, Hoogewijs MK, **De Schauwer C**, Dewulf J, de Kruif A. Transvaginal ultrasound-guided aspiration of unilateral twin gestation in the mare. Equine Vet J 2008;40(5):521-522.

Govaere J, Hoogewijs M, **De Schauwer C**, Van Loon G, de Kruif A. Incomplete placentation after twin reduction in a mare. Vet Rec 2008;163(25):747-748.

Govaere J, Hoogewijs M, **De Schauwer C**, Van Zeveren A, Smits K, Cornillie P, de Kruif A. An Abortion of Monozygotic Twins in a Warmblood Mare. Reprod Domest Anim 2009;44(5):852-854.

Govaere J, Ducatelle R, Hoogewijs M, **De Schauwer C**, de Kruif A. Case of Bilateral Seminoma in a Trotter Stallion. Reprod Domest Anim 2010;45(3):537-539.

Hoogewijs M, Rijsselaere T, De Vliegher S, Vanhaesebrouck E, **De Schauwer C**, Govaere J, Thys M, Hoflack G, Van Soom A, de Kruif A. Influence of different centrifugation protocols on equine semen preservation. Theriogenology 2010;74(1):118-126.

De Schauwer C, Meyer E, Van de Walle GR, Van Soom A. Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 2011;75(8):1431-1443

Govaere J, Vercauteren G, Coopmans C, Hoogewijs M, **De Schauwer C**, Smits K, de Kruif A. Amnion nodosum in a Belgian draught horse. Vet Rec. 2011;168(1):22-30.

De Schauwer C, Meyer E, Cornillie P, De Vliegher S, Van de Walle GR, Hoogewijs M, Declercq H, Govaere J, Demeyere K, Cornelissen M, Van Soom A. Optimization of the isolation, culture, and characterization of equine umbilical cord blood mesenchymal stromal cells. Tissue Eng Part C Methods 2011;17(11):1061-1070.

Hoogewijs M, De Vliegher S, **De Schauwer C**, Govaere J, Smits K, Hoflack G, de Kruif A, Van Soom A. Validation and usefulness of the Sperm Quality Analyzer V equine for equine semen analysis. Theriogenology 2011;75(1):189-194.

Govaere J, Maes S, Saey V, Blancke W, Hoogewijs M, **De Schauwer C**, Smits K, Roels K, Vercauteren G, de Kruif A. Uterine fibrosarcoma in a warmblood mare. Reprod Domest Anim 2011;46(3):564-566.

Hoogewijs M, Morrell J, Van Soom A, Govaere J, Johanisson A, Piepers S, **De Schauwer C**, de Kruif A, De Vliegher S. Sperm selection using single layer centrifugation prior to cryopreservation can increase thawed sperm quality in stallions. Equine Vet J 2011;43(S40):35-41.

Govaere J, Hoogewijs M, **De Schauwer C**, Van Aert M, de Kruif A. Dystocia management in mares. Intas Polivet 2011;12(II):329-339.

Hoogewijs MK, De Vliegher SP, Govaere JL, **De Schauwer C**, de Kruif A, Van Soom A. Influence of counting chamber type on CASA outcomes of equine semen analysis. Equine Vet J. 2012;44(5):542-9

De Schauwer C, Piepers S, Van de Walle GR, Demeyere K, Hoogewijs MK, Govaere JL, Braeckmans K, Van Soom A, Meyer E. In search for cross-reactivity to immunophenotype equine mesenchymal stromal cells by multicolor flow cytometry. Cytometry part A 2012;81A(4):312-323.

Spaas JH, **De Schauwer C,** Cornillie P, Meyer E, Van Soom A, Van de Walle GR. Culture and characterization of equine peripheral blood mesenchymal stromal cells. Vet J 2012, in press.

Govaere J, **De Schauwer C,** Hoogewijs M, Chiers K, Lefere L, Catry B, Roels K, van Heerden M, Broeckx S, de Kruif A. Hydrallantois in the mare – a report of five cases. Reprod Domest Anim, 2012, doi: 10.1111/j.1439-0531.2012.02013.x

De Schauwer C, Van de Walle GR, Piepers S, Hoogewijs MK, Govaere JLJ, Meyer E, Van Soom A. Successful isolation of equine mesenchymal stromal cells from cryopreserved umbilical cord blood-derived mononuclear cell fractions. Equine Vet J, 2012, minor revisions

De Schauwer C, Van de Walle GR, Meyer E, Van Soom A. Mesenchymal stem cell therapy in horses: useful beyond orthopaedic injuries? Stem Cells Dev, 2012, submitted.

De Schauwer C, Van de Walle GR, Piepers S, Hoogewijs MK, Govaere JLJ, Meyer E, Van Soom A. Comparative analysis of equine mesenchymal stromal cells isolated from alternative sources. J Tissue Eng Regen Med, submitted

Publications in national peer-reviewed journals

De Schauwer C, De Vliegher S, Kolkman I, de Kruif A, Opsomer G. Intramammaire infecties met Corynebacterium bovis bij melkvee. Vlaams Diergeneeskundig Tijdschrift 2006;75:4-10.

Kolkman I, De Vliegher S, **De Schauwer C**, Opsomer G, de Kruif A. Dealing with a dairy herd with an elevated bulk milk somatic cell count. Vlaams Diergeneeskundig Tijdschrift 2006;75(1):11-17.

Van Haver E, **De Schauwer C**, Rijsselaere T, Meyer E, Van Soom A. Toepassingsmogelijkheden van stamcellen in de diergeneeskunde. Vlaams Diergeneeskundig Tijdschrift 2008;77:219-225.

De Bock M, Govaere J, Martens A, Hoogewijs M, **De Schauwer C**, Van Damme K, de Kruif A. Torsion of the spermatic cord in a warmblood stallion. Vlaams Diergeneeskundig Tijdschrift 2008;76(6):443-446.

Smits K, Govaere J, Hoogewijs M, **De Schauwer C**, Vanhaesebrouck E, Van Poucke M, van den Berg M, Vullers T, Van Soom A. Birth of the first ICSI foal in the Benelux. Vlaams Diergeneeskundig Tijdschrift 2010:79(2):134-138.

Vanhaesebrouck E, Govaere J, Smits K, Durie I, Vercauteren G, Marten A, Schauvliege S, Ducatelle R, Hoogewijs M, **De Schauwer C**, de Kruif A. Ovarian teratoma in the mare: a review and two cases. Vlaams Diergeneeskundig Tijdschrift 2010;79(1):32-41.

Spaas JH, Gambacurta A, Polettini M, Broeckx S, Van Hoeck F, **De Schauwer C**, Van de Walle GR, Van Soom A. Purification and expansion of stem cells from equine peripheral blood, with clinical applications. Vlaams Diergeneeskundig Tijdschrift 2011;80(2):129-135.

Abstracts and proceedings in international conferences

Govaere J, Hoogewijs M, **De Schauwer C**, De Vliegher S, Duchateau L, Van Soom A, de Kruif A. Effect of artificial insemination protocol and sperm dose on pregnancy results in mares. Theriogenology 2007;68(3):505.

De Schauwer C, Piepers S, Hoogewijs MK, Govaere JLJ, Rijsselaere T, Demeyere K, Meyer E, Van Soom A. Isolation, preservation, and characterization of equine umbilical cord blood stem cells. Reprod Fertil Dev 2008;20(1):220.

Govaere J, Hoogewijs MK, **De Schauwer C**, De Vliegher S, Saey V, de Kruif A. Lack of association between low vitamin E levels and retentio secundinarum in Belgian Draught Horses (BDH) and Warmblood (WB) mares. Reproduct Domest Anim 2008;43:105.

De Schauwer C, Meyer E, Hoogewijs M, De Vliegher S, Rijsselaere T, Govaere J, Demeyere K, Van Soom A. Comparison of different methods to isolate and culture multipotent mesenchymal stromal cells from equine umbilical cord blood. Reprod Domest Anim 2009;44:100-101.

Govaere J, Hoogewijs M, **De Schauwer C**, Smits K, Vanhaesebrouck E, de Kruif A. Placental Characteristics in Belgian Draught and Warmblood Horses. Reprod Domest Anim 2009;44:105.

Hoogewijs M, Vanhaesebrouck E, De Vliegher S, Govaere JLJ, Rijsselaere T, Van Soom A, **De Schauwer C**, Smits K, de Kruif A. Effects of centrifugation protocol on sperm loss and sperm quality of equine semen. Annual conference of the American College of Theriogenologists – Albuqerque, 2009.

Hoogewijs MK, Govaere JL, Rijsselaere T, **De Schauwer C**, Vanhaesebrouck E, de Kruif A, De Vliegher S. The influence of technical settings on CASA motility parameters of frozen thawed stallion semen. 55th Annual meeting of the American Association of Equine Practitioners – Las Vegas, 2009.

Govaere J, Martens K, Vanhaesebrouck E, Hoogewijs M, **De Schauwer C**, Smits K, Roels K, Vandaele L, de Kruif A. The FoalinMare: Insights inside the Foaling Mare. Reprod Domest Anim 2009;44:79.

Govaere J, Martens K, Vanhaesebrouck E, Hoogewijs M, **De Schauwer C**, Roels K, Vandaele L, de Kruif A. The Foalinmare: insights inside the foaling mare; an animated educational tool for enhancing veterinary training. 55th Annual meeting of the American Association of Equine Practitioners – Las Vegas, 2009.

Govaere J, Martens K, Vanhaesebrouck E, Hoogewijs M, **De Schauwer C**, Roels K, Vandaele L, de Kruif A. The Foalinmare : insights inside the foaling mare; an animated educational tool for enhancing veterinary training. GMA-Workshop Tiermedizin und 4. eLearning-Symposium der deutschsprachigen tiermedizinischen Bildungsstätten, 2009 October 8th-10th Freiburg.

Hoogewijs M, Piepers S, Govaere J, **De Schauwer C**, Smits K, de Kruif A, Van Soom A. Influence of extender on the storage of equine semen at different temperatures. Reprod Domest Anim 2010;45S(3):60.

Hoogewijs MK, De Vliegher S, Govaere JL, **De Schauwer C**, de Kruif A, Van Soom A. Need for further improvement of SQA-Ve software for a univocal analysis of the quality of frozen thawed equine semen. 7th Biennial meeting of the Association for Applied Animal Andrology – Sydney, 2010.

Govaere J, Hoogewijs M, **De Schauwer C**, Smits K, Vandaele H, Van Loon G, de Kruif A. Twisting of mummified remains around the umbilical cord as a potential risk of delayed twin reduction in the mare. Reprod Domest Anim 2010;45S(3):59.

Govaere J, Martens K, Hoogewijs M, **De Schauwer C**, Roels K, Smits K, de Kruif A. FoalinMare: Kisraklarda Doğum. IVth Veteriner Jinekoloji Kongresi (Uluslararasi Katilimli) 4-7 Kasim, Belek, Antalya 2010;22-23.

De Schauwer C, Van Soom A, Van de Walle GR, Demeyere K, Meyer E. Immunophenotypic characterization of equine mesenchymal stromal cells using flow cytometry. Reprod Domest Anim 2011,46S(3):97-98.

Hoogewijs M, De Vliegher S, Govaere J, **De Schauwer C**, Van Soom A, de Kruif A. Analysing stallion semen: factors affecting CASA outcomes. Reprod Domest Anim 2011;46S(3):70.

Govaere J, Hoogewijs M, **De Schauwer C**, Roels K, Valcke M, de Kruif A. The FoalinMare dvd: an UGhent educational project with a worldwide audience. 3th dag van de Onderwijs Innovatie, UFO Ghent 2011, February 9th.

Hoogewijs M, Morrell J, Van Soom A, Govaere J, Johannisson A, Piepers S, **De Schauwer C**, de Kruif A, De Vliegher S. Sperm selection using single layer centrifugation prior to cryopreservation can increase post-thaw sperm quality in stallions. 57th Annual meeting of the American Association of Equine Practitioners – San Antonio, 2011.

De Schauwer C, Van de Walle GR, Meyer E, Van Soom A. Cultivation of mesenchymal stromal cells from cryopreserved mononuclear cells isolated from equine umbilical cord blood. Reprod Domest Anim 2012;47S(4):590.

Hoogewijs M, Rijsselaere T, Piepers S, Roels K, Govaere J, **De Schauwer C**, Van Soom A. Methylformamide and glycerol as succesful combination for canine semen preservation. Reprod Domest Anim 2012;47S(4):442-443.

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