

Purification and expansion of stem cells from equine peripheral blood, with clinical applications

Opzuivering en aanrijking van stamcellen uit het perifere bloed bij het paard met klinische toepassingen

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ABSTRACT

Equine peripheral blood (ePB) can be used as a source of stem cells (SCs) in horses, both for research and for practical purposes. A relatively low volume of ePB is sufficient for the purification and expansion of the SCs. The identification of the SCs is performed by demonstrating the presence (CD34, CD90, CD105 and CD117) or absence (CD14) of specific markers on the cell surface by means of fluorescent staining, followed by Fluorescence Activated Cell Sorting (FACS) for sorting out the desired population of SCs. The entire process of SC isolation and enrichment from ePB typically takes three days, after which the enriched SC sample can be sent back to the patient for clinical application. The two most common clinical applications of SCs from ePB will be demonstrated with two field cases. The first case presents a lesion of the body of the suspensory ligament in a 13-year-old warmblood mare and the second case describes a bacterial ulcerative keratitis in a 20-year-old warmblood gelding.

SAMENVATTING

Zowel in het kader van de wetenschap als voor praktische doeleinden kan perifeer bloed van het paard als bron voor stamcellen (SCs) gebruikt worden. Een relatief laag volume perifeer bloed is voldoende om SCs op te zuiveren en aan te rijken. De identificatie van SCs kan uitgevoerd worden door de aanwezigheid (CD34, CD90, CD105 en CD117) of afwezigheid (CD14) van specifieke markers op het celoppervlak die aangetoond worden aan de hand van een immunofluorescentiekleuring, gevolgd door *fluorescence activated cell sorting* (FACS) om de gewenste celpopulatie te sorteren. Het volledige proces van SC-isolatie en aanrijking duurt gemiddeld drie dagen. Daarna kan het aangerijkte SC-mengsel teruggezonden worden naar de patiënt. Twee interessante toepassingen van SCs uit het perifeer bloed worden geïllustreerd. Het eerste geval handelt over een laesie in het middelste aspect van de *musculus interosseus medius* bij een dertienjarige warmbloedmerrie. Het tweede geval gaat over bacteriële ulceratieve keratitis bij een twintigjarige warmbloedruin.

INTRODUCTION

Stem cells (SCs) are defined as cells that show self-renewal either with or without differentiation, depending on the symmetry of the division (Donovan and Gearhart, 2006). The different types of SCs and their environments, the niches, determine the differentiation pathways they can follow (Mezey *et al.*, 2000). Multipotent SCs, such as mesenchymal SCs (MSCs) and hematopoietic SCs (HSCs), can only differentiate into a limited number of tissues in the adult individual (Roelandt *et al.*, 2010). To date, there have been reports of differentiation of equine MSCs into cartilage (Hegewald *et al.*, 2004), bone (Vidal *et al.*, 2006), fat (Koch *et al.*,

2007), muscle (Martinello *et al.*, 2009) and tendon (Smith, 2008). Hematopoietic SCs have the ability to form all the different types of blood cells (Akashi *et al.*, 2000). Pluripotent SCs, on the other hand, are able to differentiate into every cell type from the three germ layers of the embryo (endoderm, mesoderm, and ectoderm). Embryonic SCs (ESCs) are a typical example of such pluripotent SCs, and to date, six lines of ESCs have been described in the horse, although their pluripotency remains to be demonstrated *in vivo* (Donovan and Gearhart, 2006; Paris and Stout, 2010).

The use of SC therapy in horses is a hot topic (Van Haver *et al.*, 2008). Mesenchymal SCs (MSCs) obtained from the bone marrow have frequently been des-

cribed as a novel treatment for tendinopathies (Crovace *et al.*, 2007; Smith *et al.*, 2003; Smith, 2006; Smith, 2008; Violini *et al.*, 2009). In addition, the administration of MSCs has been proposed as a promising treatment for other diseases such as arthritis (Wilke *et al.*, 2007), bone fractures (Vidal *et al.*, 2006), hepatic disorders (Petersen *et al.*, 1999), pancreatic dysfunction (Santana *et al.*, 2006), myocardial pathologies (Chen *et al.*, 2006), epithelial defects in the respiratory system (Nowak and Fucks, 2009) and even neoplastic processes (Gratwohl *et al.*, 2007).

Besides bone marrow, other possible sources of MSCs have been described, including fat, liver, umbilical cord (Wharton's jelly), fetal pancreas and equine peripheral blood (ePB) (Campagnoli *et al.*, 2001; Ericsson *et al.*, 2000; Hu *et al.*, 2003; Kuwana *et al.*, 2003; Zuk *et al.*, 2001; Zuk *et al.*, 2002; Zvaifler *et al.*, 2000). Equine peripheral blood represents an interesting source of SCs because of the low invasivity, ease of harvesting, and low pain levels involved in the harvesting procedure. In this report, two clinical cases are presented where SCs from the ePB were used to treat a suspensory ligament desmitis (case one) and a bacterial ulcerative keratitis (case two). The salient findings were that these two clinical cases, each with a long-lasting pathology which did not respond to conventional treatment, showed a significant improvement after treatment with autologous SCs.

LABORATORY TECHNIQUES

A venous blood sample (5-7 ml in EDTA anticoagulants) was taken from the patient and transported to the laboratory at a maximum temperature of 7°C. Upon arrival at the laboratory, the white blood cell fraction was immediately isolated using a Ficoll-Paque gradient, following the manufacturer's instructions (Pharmacia Biotech AB, Uppsala, Sweden), and washed with phosphate-buffered saline (PBS) to remove unwanted red blood cells. The white blood cell fraction was incubated for 72 hours in a sterile incubator at 37.5°C and 5% CO₂ with granulocyte- and monocyte-colony stimulating factor (G-CSF & M-CSF, Sigma, Missouri, USA) and interleukine (IL) 1, 3 and 6 (patent number ThankStem: 07 820 134.0-1222). Since SCs are fast dividing cells, there was a sufficient number of SCs after 72 hours in comparison to the number of differentiated monocytes.

Subsequently, the cells were divided in two groups and incubated simultaneously with saturating amounts of the fluorescently labeled human antibodies CD90/CD117 and CD14/CD34/CD105, respectively, for 30 minutes at room temperature (RT). The antibodies used were CD34-PerCp, CD90-PE, CD105-FITC, CD117-APC (all from BD Biosciences, San Jose, USA), and CD14-PE (Biolegend, San Diego, USA). This mixture was then sorted with a fluorescent activated cell sorter (FACSaria II cell sorter, BD Biosciences, San Jose, USA) to isolate SCs from the ePB. The markers used to characterize the equine SCs have all been previously described to cross react with equine

Table 1. The isolated stem cells (SCs) from equine peripheral blood (ePB) were positive for hematopoietic SC (HSC) markers CD34 and CD117, and for mesenchymal SC (MSC) markers CD90 and CD105, and negative for a differentiated blood cell marker, CD14 (Guest *et al.*, 2009; Martinello *et al.*, 2009; Hoynowski *et al.*, 2007).

Marker	HSCs	MSCs	SCs from ePB
CD14	-	-	-
CD34	+	-	+
CD90	-	+	+
CD105	-	+	+
CD117	+	-	+

antigens. Their presence or absence in the isolated stem cell population is indicated in Table 1. Indeed, SCs from the ePB isolated and used for the case studies were clearly positive for the MSC markers CD90 (Martinello *et al.*, 2009) and CD105 (Hoynowski *et al.*, 2007), and for the HSC markers CD34 (Martinello *et al.*, 2009) and CD117 (Martinello *et al.*, 2009), and they were negative for CD14, a marker used for differentiated hematopoietic cells (Guest *et al.*, 2008; Ibrahim *et al.*, 2007). In addition, the sorting of these cells resulted in the elimination of the unwanted differentiated cells, and thus a rather pure isolation of a SC population with hematopoietic and mesenchymal properties was obtained.

After sorting, the cells were washed with PBS and resuspended in a PBS solution, supplemented with 5µM gentamycin. Two sterile 15ml tubes, each with a 5ml suspension of 125 x 10³ SCs (or 25 x 10³ SCs/ml) and 500 x 10³ SCs (or 100 x 10³ SCs/ml), were prepared for intravenous and local application, respectively. The cells were then transported back to the patient as soon as possible at 7°C in a cooling box.

FIELD STUDIES

Case report 1: a suspensory ligament desmitis treated with ePB-derived SCs

History

A 13-year-old Belgian warmblood mare was presented with lameness and a swelling on the right front leg. After a thorough examination, the swelling was situated in the mid-region of the palmarolateral aspect of the canon bone. The mare was lame in the right front leg on a straight line, and in the outside leg on the left circle on a soft surface. On ultrasound examination, heterogenic regions were noted in the mid-region of the suspensory ligament (*musculus interosseus medius*, MIM) of the right front leg. Other structures visualized on the ultrasound were a blood vessel (BV), the distal check ligament (DCL), the deep digital flexor tendon (DDFT), and the superficial digital flexor tendon (SDFT), ordered from dorsal to palmar (Figure 1a). The horse had been treated for three weeks with conservative therapy. The oral application of anti-inflam-

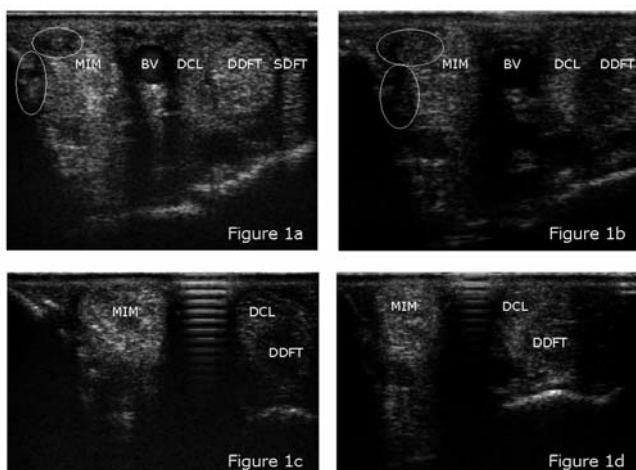


Figure 1. Transversal ultrasound images of the midregion of the suspensory ligament (*musculus interosseus medius*, MIM) of the right front leg of a 13-year-old Belgian warmblood mare. Ultrasound images: (a) on the first day of clinical signs, (b) on the day of SC therapy, (c) five weeks post-therapy and (d) nine weeks post-therapy. The lesions are encircled. BV: blood vessel; DCL: distal check ligament; DDFT: deep digital flexor tendon; SDFT: superficial digital flexor tendon.

matory drugs (Fenylbutazon, 2g/day) for two weeks in combination with cooling therapy four times a day for three weeks with ice cold water for ten minutes on the swollen leg did not improve the symptoms (Figure 1b).

Stem cell therapy

Because this horse was clearly suffering, the owners decided to try SC therapy. A blood sample was collected and, after isolation and enrichment of the autologous SCs, 500×10^3 cells in 5ml were injected locally into the lesion with a sterile 2.5ml syringe and 20G needle with multiple punctures. This was performed in a sterile way and ultrasound guided in order to localize the area of the lesion. After that, the owners were advised to immobilize the horse for ten days, followed by walking exercises for three months and gradually increasing the labor in the following three months to a competitive level.

Results

Five weeks after the SC injection the swelling had disappeared and the horse showed no more signs of lameness. Moreover, the lesion was filled with tendon tissue, although there was still some heterogeneity noticeable at the edges of the lesion site (Figure 1c). Nine weeks post-injection, there were no more signs of the lesion on ultrasonography (Figure 1d). After the following nine weeks of walking exercises (i.e. 18 weeks post-injection), the mare started trotting and cantering gradually. At six months post-injection, the horse reached the original level of competition in show jumping with no recurrence of the symptoms up to the present (one year post-injection).



Figure 2. Photos of a 20-year-old horse with a bacterial ulcerative keratitis visualize the inflamed and ulcerated right eye: (a) on the day of the SC therapy, (b) two weeks post-therapy, and (c) three months post-therapy. The general condition of the horse under identical management circumstances is pictured: (d) on the day of stem cell therapy and (e) three months after the day of SC therapy.

Case report 2: bacterial ulcerative keratitis treated with ePB-derived SCs

History

A 20-year-old Italian warmblood gelding was presented with a painful, inflamed eye with extensive tear flow. A corneal ulcer was assumed, based on the results of the fluorescein eye stain and on the clouded region that was detected in the centro-ventral part of the eye. The results of bacteriological examination had shown that *Pseudomonas aeruginosa* was the main causing agent of this pathological eye condition. The horse had been treated for six months with different kinds of antibiotics (gentamycin, tobramycin, tetracyclines, chloramfenicol and colistine), non-steroid anti-inflammatory drugs (flunixin meglumine, sodium diclofenac, and sodium flurbiprofen dehydrate) and other farmaceutics (acetylcysteine, atropin sulfate, riboflavin, xanthopterin and d- α -tocopherol) with only a minimal improvement of the symptoms. In addition, the local application of miconazol was initiated, even though no yeast or fungi had been isolated after a scraping sample. Because this ulcer appeared resistant against all the possible conservative therapies, the owners chose for surgical intervention. The ulcer was scraped and swabbed with iodide tincture (50% concentration). Nevertheless, the inflammation of the eye and the ulcer remained present at the centroventral part of the eye in spite of the different treatments for an exceedingly long period (Figure 2a).

Stem cell therapy

Because there was so little improvement of the pathological condition, the owners decided to try SC the-

rapy as a last resort. A one-time injection both in the jugular vein and in transverse facial artery (with a 27-gauge butterfly catheter) was performed. The intravenous injection with 125×10^3 SCs in 5ml was performed because of the poor general condition of the horse. The intra-arterial injection, also with 125×10^3 SCs in 5ml, was performed in order to reach the internal structures of the eye. In addition, a local application of ePB-derived SCs was initiated with an eye drop formulation of 500×10^3 SCs in 5ml, three times a day for ten consecutive days. Resuspension of the sedimented cells was performed by gently turning the bottle a couple of times a day. After seven days, clouded areas were visible in the cell suspension, probably because of cell death, and therefore the eye drop application was stopped to avoid an inflammatory reaction to the necrotic cells.

Results

Already two weeks later, positive effects were visible. The inflammation was starting to disappear and the tear flow was decreasing. The size of the ulcer was clearly reduced and remained stable (Figure 2b). Three months after the SC treatment, the eye ulcer was almost invisible and the inflammation had disappeared, along with the pain and irritation (Figure 2c). Overall, the horse was in much better general condition, as is evident from a comparison of the photo in Figure 2d, which was taken before SC therapy, and the photo in Figure 2e, which was taken three months after SC therapy.

DISCUSSION

Stem cells (SCs) represent a very promising treatment for certain types of degenerative or traumatic diseases because of their plasticity and differentiation capacities. Their use in equine veterinary medicine has been intensively studied in recent years, and their regenerative effect, mainly with tendon and ligament injuries, has been described in different independent *in vivo* studies (Crovace *et al.*, 2007; Smith *et al.*, 2003; Smith, 2008).

In order to be classified as SCs, cells have to fulfill several requirements that are clearly defined for human SCs (International Society for Cellular Therapy (ISCT), Dominici *et al.*, 2006), although no such strict definitions for veterinary stem cells have been established to date. It is generally accepted that mesenchymal stem cells (MSCs), including those of equine origin, must express several typical MSC markers, and must also have the capacity to differentiate into adipocytes, chondroblasts and osteoblasts (Dominici *et al.*, 2006). The characterization of equine MSCs is mostly described by the presence of CD90 and CD105, and the lack of CD14 and CD34 (Guest *et al.*, 2009; Hoynowski *et al.*, 2007; Martinello *et al.*, 2009). In the SC population used in this study, the sorted cells were positive for 2 typical MSC markers, CD90 and CD105, and they were also positive for two typical hemato-

poietic stem cells (HSC) markers, CD34 and CD117 (Table 1). This indicates the commitment of these SCs towards the MSC lineage, since in the study by Martinello *et al.* (2009) the MSC were also CD117 positive. In order to state this with a higher level of certainty, differentiation into different mesenchymal cell types (chondroblasts, osteoblasts and adipocytes) of the mesodermal germ layer should be studied as described by the ISCT for human SCs (Dominici *et al.*, 2006). Up to the present, however, no such experiments have been done with the sorted ePB-derived SCs. The 'stemness' of these cells is based solely on the expression of certain SC markers. In a study by Koerner *et al.* (2006), progenitor cells isolated from the ePB were clearly able to differentiate into osteoblasts and adipocytes. Giovannini *et al.* (2008) also described multilineage differentiation potential, including differentiation into chondroblasts of ePB progenitor cells.

In human peripheral blood, approximately 28 cells per 10^6 peripheral blood mononuclear cells (MNC) are SCs (Bian *et al.*, 2009). In horses, the number of MNC (lymphocytes and monocytes) varies around 3.41×10^6 /ml blood (Cebulj-Kadunc *et al.*, 2003). Extrapolating the data from humans to horses, we estimate that around 95 SCs/ml blood are present. In the present study, 5ml of peripheral blood from the patients was used, containing approximately 475 SCs (5×95). These stem cells were then expanded for three days with G-CSF, M-CSF, and IL 1, 3 and 6 (patent number ThankStem: 07 820 134.0-1222), to obtain a sufficient number (ranging from 500 to 750×10^3 SCs) for therapeutic use.

For the regeneration of tendon tissue, bone marrow is the most common source of equine MSCs, and it is for this reason that such therapies are the best studied until now (Smith *et al.*, 2003; Smith, 2006; Smith, 2008) and, consequently, the differentiation of MSCs from bone marrow into adipocytes, osteoblasts and chondroblasts has been thoroughly studied as well (Hegewald *et al.*, 2004; Longobardi *et al.*, 2001; Vidal *et al.*, 2006; Wilke *et al.*, 2007). Since the goal of SC therapy is to reach '*restitutio ad integrum*' (restitution of the original, functional state), these findings are very promising and, all the more so, because they are supported by several *in vivo* studies. In a study by Hertel (2001), 84% of the horses with a suspensory ligament desmitis treated with bone marrow-derived SCs returned to full work, in contrast to the conservative treatment, whereby only 15% of the horses reached the same level as before. It must be noted, however, that in this study no information was given on the frequency of forelimb and hindlimb problems, which have different prognoses. In another study, the beneficial effects of SC therapy were evaluated in horses suffering from superficial digital flexor tendinitis (Smith, 2008). This study found that 82% of the horses treated with MSCs performed at their original level without re-injury in the next year (Smith, 2008), whereas only 44% of the horses treated with conservative therapy did so (Dyson, 2004). Here it should be mentioned that the documentation from the control group spanned a lon-

ger time frame post-treatment (two years) compared to the study by Smith (2008).

In the first case study of the present report, SCs from ePB were used to treat a horse suffering from a suspensory ligament desmitis. Although the cell injection happened quite late in this case (after three weeks of conservative therapy), the lesion soon showed very promising improvement on ultrasound, and six months later the horse was clinically normal, as she was show jumping again and showed no signs of recurrence. Still, it is important to point out that we do not know if this horse would have shown the same improvement with a conservative therapy for five more weeks. In order to prove the effectiveness of SCs, we should have used a standardized model with a similar induced lesion in a significant number of horses. Also, a double blind setup with half of the horses treated with SCs and the other half with PBS and 5µM gentamycin (which is used as a carrier to inject the stem cells) as a control group, would have been an appropriate experimental setup. On the other hand, there are the ethical questions that arise from these kinds of clinical trials. Since we used a spontaneously occurring tendon injury, a control group with exactly the same lesion was not possible. Moreover, the horse went into competition again, which eliminated the option of histological examination.

In the second case, SC therapy was applied to a horse suffering from a painful bacterial ulcerative keratitis. For six months, different therapies were tried without any success, for which reason, SCs were used as a last resort. The use of SCs from ePB as a possible treatment for corneal lesions or ulcers has not been described in horses until now, although the use of eye SCs and bone marrow-derived MSCs has been described previously as a treatment for corneal lesions in a large number of different mammal species such as mice, rats, rabbits, horses and humans (Girolamo, 2009; Jiang *et al.*, 2010; Koizumi *et al.*, 2009; Shimazaki *et al.*, 2002; Ueno *et al.*, 2007). In the present case, the 20-year-old horse with the corneal ulcer showed a positive evolution after local, intravenous and intra-arterial SC therapy, although further scientific research is needed in order to understand their *modus operandi* and to be able to propose a standardized protocol for using equine SC therapy in the treatment of eye problems. In human medicine, it has been pointed out that the intravenous application of adipose MSCs improved the general condition and decreased the symptoms (pain, coordination problems and loss of cognition) of three multiple sclerosis (MS) patients (Riordan *et al.*, 2009). Since there were no control groups, the placebo effect of the intravenous injections cannot be ruled out. Therefore, the value of the intravenous application of SCs in order to improve the general condition of this horse is questionable.

In conclusion, these reports describe long-lasting pathologies that did not respond to conventional medical treatment, for which reason SCs from ePB were used as a novel therapy. Both horses showed significant improvement after this treatment, a fact which indicates the great potential of these SCs. Moreover, ePB is

a very interesting source of SCs because of the low invasivity, ease of harvesting and low pain levels involved in the harvesting procedure (Kassis *et al.*, 2006; Zvaifler *et al.*, 2000). Still, many questions remain and more basic scientific research is certainly needed to fully unravel the regenerative effects of SCs in veterinary medicine in general, and in equine regenerative therapy in particular.

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Mededeling



O₂ 4 U² – EVECCS 2011

Het congres voor de dierenarts en paraveterinair met interesse in de Spoedeisende en Intensieve Zorg Diergeneeskunde

O₂ 4 U² is een knipoog naar de essentiële rol van zuurstof bij het leven en hoewel de nadruk van het EVECCS Congress 2011 ligt op de rol van zuurstof in relatie tot de Spoedeisende en Intensieve Zorg Geneeskunde, zal er een uitgebreid scala aan andere ECC-onderwerpen de revue passeren.

Pre-congress Dag

“Wat als...” uw patiënt niet gereageerd heeft op de ingestelde behandeling, maar u aan het eind van het boekje bent? Met deze frustrerende situatie als gegeven, hebben wij onze sprekers bereid gevonden om hun tips en trucs met u te delen.

Hoofdsprekers

Een lange lijst van nationaal en internationaal bekende sprekers zal meer dan 60 lezingen verzorgen.

Gezelschapsdieren: Steve Haskins (USA), Nadja Sigrist (CH), Yves Moens (AT)

Paard: Bruce McGorum (UK), Gunther van Loon (BE)

Paraveterinair: Angel Rivera (USA)

Workshops

Een keur aan workshops zal aan alle drie de stromingen worden aangeboden.

Gezelschapsdieren: Catheters & Tubes, Cytologie bij de spoedpatiënt, Echografie bij de spoedpatiënt, Spoed procedures bij vogels en exoten

Paard: Echografie Thorax/Hart, Cytologie bij de spoedpatiënt

Paraveterinair: CPCR

Scientific Abstracts

EVECCS verwelkomt short communications voor het 10^e EVECCS Wetenschappelijke Programma. Voor verdere details verwijzen wij u naar de website:

www.EVECCS2011.org

EVECCS 2011 wordt mede mogelijk gemaakt met de hulp van haar partners **Royal Canin** en **Braun**