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Blood Derived Stem Cells: An Ameliorative Therapy in Veterinary Ophthalmology

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Stem cell technology has evoked considerable excitement among people interested in the welfare of animals, as it has suggested the potential availability of new tools for several pathologies, including eye disease, which in many cases is considered incurable. One such example is ulcerative keratitis, which is very frequent in horses. Because some of these corneal ulcers can be very severe, progress rapidly and, therefore, can be a possible cause of vision loss, it is important to diagnose them at an early stage and administer an appropriate treatment, which can be medical, surgical, or a combination of both. The therapeutic strategy should eradicate the infection in order to reduce or stop destruction of the cornea. In addition, it should support the corneal structures and control the uveal reaction, and the pain associated with it, in order to minimize scarring. In this study, we address how stem cells derived from peripheral blood can be used also in ophthalmological pathologies. Our results demonstrate that this treatment protocol improved eye disease in four horse cases, including corneal ulcers and one case of retinal detachment. In all cases, we detected a decrease in the intense inflammatory reaction as well as the restoration of the epithelial surface of the central cornea.

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The considerable excitement surrounding the field of stem cell research is based on the unique biological properties of these cells and their capacity to self-renew and regenerate tissue and organ systems. Hematopoietic stem cells (HSCs) are currently used in clinical stem cell transplantation. In addition, they hold great promise for future regenerative medicine, tissue repair, and gene therapy. However, the molecular mechanisms controlling the self-renewal/proliferation of HSCs or the commitment along hematopoietic lineages are still not completely understood. Although, to date, cell fate conversions in adults are rare occurrences, it is conceivable that the improvement of the methods and experimental approaches devoted to the isolation and ex vivo expansion of stem cells before reinfusion will improve these phenomena. For instance, HSCs are a safe and accessible source of stem cells, easily harvested for clinical use. In addition, HSCs avoid the immunological problems associated with allograft, as the donor is the recipient. Therefore, studies intended to optimize the ex vivo expansion of HSCs and explore their differentiative potential have clear therapeutic relevance with regard to increasing the flexibility and applicability of tissue regeneration strategies. Indeed, regeneration of tissues with endogenous stem cells clearly holds promise for a novel class of therapies that are able to treat a multitude of serious diseases and injuries. Several recent in vivo studies suggest that adult multipotent blood-derived stem cells (BDSCs) (Spaas et al., 2011) are capable of differentiation into a variety of adult cell types including neurons. Furthermore, a number of experimental observations have shown that these cells can spontaneously repopulate different organs such as muscle, bone, liver or heart and generate differentiated cells in response to acute or chronic organ damage. All these observations, which suggest that these stem cells are capable of contributing to tissue regeneration (Donovan and Gearhart, 2001), have ignited significant interest in the possibility that cell therapy could be employed for the repair of damaged non-hematopoietic tissues. It is likely that the circulation of large quantities of these cells that have been injected into the peripheral blood might favor their lodging in severely damaged organs. Indeed, injection of stem cells into peripheral blood represents a non-invasive and potentially effective approach to easily obtain large quantities of stem cells capable of lodging into damaged tissues (Gratwohl et al., 2007). The discovery of the therapeutic potential of stem cells offers

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new opportunities for the treatment of incurable diseases. Although stem cells offer great opportunities for repair of the nervous system and the eye, their clinical use necessitates that we first gain an understanding of their proliferation, migration, differentiation, immunogenicity, and establishment of functional cell contacts. It will also be necessary to produce these cells under conditions that meet appropriate safety and effectiveness standards. Our current understanding of the critical factors affecting stem cell behavior remains limited. Rapid progress is being made, and some of the first applications of stem cells to wound repair in human eyes have produced successes that offer hope for the use of stem cells in other ophthalmological conditions.

In the veterinary field, the use of stem cells is gaining importance for exploring the potential of regeneration medicine in the treatment of several diseases. The use of stem cell therapy in horses is a hot topic nowadays (Van Haver et al., 2008; Paris and Stout, 2010). Different sources have been described as suitable for obtaining stem cells: Bone marrow, fat, liver, umbilical cord blood, and fetal pancreas (Petersen et al., 1999; Erices et al., 2000; Lin et al., 2000; Campagnoli et al., 2001; Zuk et al., 2001, 2002; Hu et al., 2003; Kuwana et al., 2003; Koch et al., 2007). Another possible source of equine stem cells is the peripheral blood (Giovani et al., 2008; Spaas et al., 2011), which represents an interesting source of stem cells because the process of taking the blood is fairly easy, not very invasive, and causes minimal pain to the animal. At present, the most common sources of stem cells are animal adipose tissue or bone marrow. In both cases, sampling is very invasive and protocols do not provide a homogeneous population of stem cells (Blatt et al., 2005; Cho et al., 2005).

Melting corneal ulcers in horses are potentially vision and globe threatening and therapy must be aggressive (Severin, 1976). These complicated ulcers may lead to impaired vision because of corneal scaring or anterior synechia, or lead to the loss of the eye because of endophthalmitis, glaucoma, phtisis bulbi, or a combination of these. In cases of melting ulcers where the stromal breakdown is quickly halted and the area involved is relatively small, the extent of the end-stage fibrosis is usually quite limited. The visual outcome for such corneal ulcers should be over 90%, and the globe survival over 95% (Olive, 2005). However, more extensive lesions result in a permanent leukoma, which may interfere with vision. In cases where the melting progresses and causes an iris prolapse, the visual outcome decreases to 40%, and globe survival to 67% (Olive, 2005). Finally, in cases where the eye is irreversibly damaged before beginning treatment, failure of stromal regeneration, end-stage corneal fibrosis and phtisis bulbi are inevitable sequellae and enucleation should be considered (Shimazaki et al., 2002; Olive, 2005).

Here we report four cases of eye injuries in horses, including corneal ulcers and one case of retinal detachment treated with the stem cells from peripheral blood. We injected BDSCs either directly into the lesions or intravenously in the same animals, thus obtaining a fast and efficient tissue/cell repair.

The important findings of this study are that these clinical cases do not respond to conventional treatment while they show a significant improvement after treatment with autologous stem cell therapy.

Materials and Methods

Stem cell expansion

Blood samples (5–7 ml in EDTA) were collected from the lower limbs of animals. The nucleated blood cell fraction was isolated by ammonium chloride incubation (dilution 1:3 in NH_4Cl 1 M), centrifuged at 400*g* and washed several times with phosphate buffered saline, pH 7.2 (PBS) (Oxoid, Sigma-Aldrich, St. Louis, MO) to remove the majority of erythrocytes. Cells were then resuspended in 5 ml of PBS and incubated for 72 h at 37°C in the presence of 50 nM macrophage colony-stimulating factor (M-CSF, Sigma-Aldrich, St. Louis, MO), and 5 μM gentamicin sulfate (Patent n° 07 820 1340-1222).

Flow cytometry analysis

For cytofluorimetric analyses, the cells were incubated with saturating amounts of fluorescently labeled human antibodies against stem cell surface markers (which cross-react with horse antigens) alone or in combination during 30 min at room temperature (RT). The antibodies used were CD90-PE, CD117-APC, CD34-PerCp, CD105-FITC, SOX2-APC, Oct3/4-APC, and Nanog-APC, all from BD Biosciences, San Jose, CA, and CD14-PE, which was purchased from Biolegend, San Diego, CA. This mixture was then sorted with a fluorescent activated cell sorter (FACSAria II cell sorter, BD Biosciences) to isolate the peripheral blood stem cells. Finally, the cells were washed again with PBS and, at this stage, were ready to be transported back to the animals at 7°C and usually within a time frame of one week (after the original blood sample collection) for local or intravenous injection, depending on the pathology.

Animal model

An official statement confirming the owner's consent was required to enroll the animals in this study. Four eyes of four animals with corneal ulcers (1 female, 3 males; median age ranging from 7 to 20 years) and two eyes of one animal (2-year-old stallion) with retinal detachment were studied.

Case 1: A 20-year-old gelding presented an inflamed eye with excessive lacrimation. A clouded region in the centroventral part of the eye also suggested a corneal ulcer. The results of bacteriological examination showed that *Pseudomonas aeruginosa* was the main causing agent of this pathological eye condition.

Case 2: A 7-year-old German mare (used for dressage) was affected by immune-mediated keratitis with vascularization, edema, and an intense inflammatory response.

Case 3: A 12-year-old gelding (jumping horse) was stung in its cornea (right eye) by a hornet. At the initial visit, his left upper eyelid was swollen and severe chemosis and subconjunctival hemorrhage were evident in this eye.

Case 4: A 13-year-old gelding with a corneal ulcer appeared as a complication of equine recurrent uveitis (ERU).

Case 5: A 2-year-old stallion presented with a retinal detachment. After some days of miosis and total bilateral blindness, a diagnosis of bilateral retinal detachment (incomplete in the right eye, almost complete in the left eye) was made. The possible causes included infective, congenital, metabolic, toxic, immune-mediated, and idiopathic.

Identification of corneal ulceration by the use of fluorescein staining

Fluorescein dye was used to detect corneal integrity. Fluorescein was used at concentrations up to 2.0% alkaline solution to check for a corneal leakage (i.e., Seidel test) (Strubbe and Gelatt, 1999; Brooks, 2004).

Results

Isolation of peripheral blood derived Stem Cells

In order to isolate stem cells from peripheral blood, we incubated nucleated blood cells from each horse with M-CSF for 72 h as described in the Materials and Methods section. Within this period, stem cell receptors were expressed on the surface of some cells. Since they were fast-dividing cells, there were a sufficient number of stem cells after 72 h for sorting. In addition, such sorting resulted in the elimination of differentiated granulocytes and monocytes and, hence, allowed us to obtain rather pure stem cells. The markers used to obtain the peripheral blood stem cells are shown in Table 1 and their

TABLE 1. Markers used to isolate and characterize blood-derived stem cells

Marker	Hematopoietic stem cell (HSC)	Mesenchymal stem cell (MSC)	Blood-derived stem cell after sorting
CD14	−	−	−
CD34	+	−	−/+
CD90	+	+	+
CD105	−	+	+
CD117	+	−	+
Sox2	−	−	−/+
Oct3/4	−	−	−/+
Nanog	−	−	−/+

presence or absence on the different stem cell populations is indicated. Indeed, the peripheral blood stem cells isolated and used for all cases were all negative for CD14, a marker used for differentiated hematopoietic cells (Kern et al., 2006) and expressed very little CD34, a HSC marker, while they were clearly positive for CD90, CD105, and CD117, which were also expressed in MSCs and/or HSCs (Table 1). Furthermore, a small fraction (about 15%), after sorting, showed the presence of stem cell embryonic markers such as Sox2, Oct3/4, and Nanog (Table 1).

Stem cell therapy

BDSCs were collected from the same animals as described in the Materials and Methods section and, upon isolation of the autologous peripheral blood stem cells, these were injected via the ophthalmic artery in the horses.

Before all of the clinical applications, the puncture locations were surgically prepared with iodide solution and alcohol. For the conjunctiva injection, 21 Gauge (G) needles measuring 40 mm were used. Furthermore, the ophthalmic intra-arterial injection of the eye was carried out with a small catheter of 23 G, 30 cm. Corneal lesions, ulcers, and inflammation, as well as equine recurrent uveitis can be treated with stem cells, not only locally in the eye artery, using a small catheter (Fig. 1a,b), but also intravenously and by the local application of an eye drop formulation used three times daily for two weeks and/or subconjunctival injection (Fig. 1c,d).

Case 1: The horse had been treated for six months with different kinds of antibiotics (gentamycin, tobramycin, tetracyclines, chloramfenicol, and colistine), non-steroid anti-inflammatory drugs (flunixine meglumine, sodium diclofenac, and sodium flurbiprofen dehydrate), and other substances (acetylcysteine, atropine sulfate, riboflavin, and d-α-tocoferol) with only a minimal improvement of the symptoms. In addition, xantopterin and miconazol were applied locally, even though no yeast or fungi were isolated after a scraping sample. Because this ulcer appeared to be resistant to all the possible conservative therapies, the owners opted for surgical intervention. The ulcer was scraped and swabbed with iodide tincture (50% concentration). After the different treatments, the eye was still very inflamed and the ulcer was still present at the centro-ventral part of the eye (Fig. 2a). For this reason, it was treated with one administration of BDSCs intravenous (IV) and local instillations of the animal's own stem cells (as described above). Two weeks later, we observed a decrease in inflammation and lacrimation (Fig. 2b). Furthermore, the ulcer size was clearly reduced and stable. After three months, the ocular ulcer was

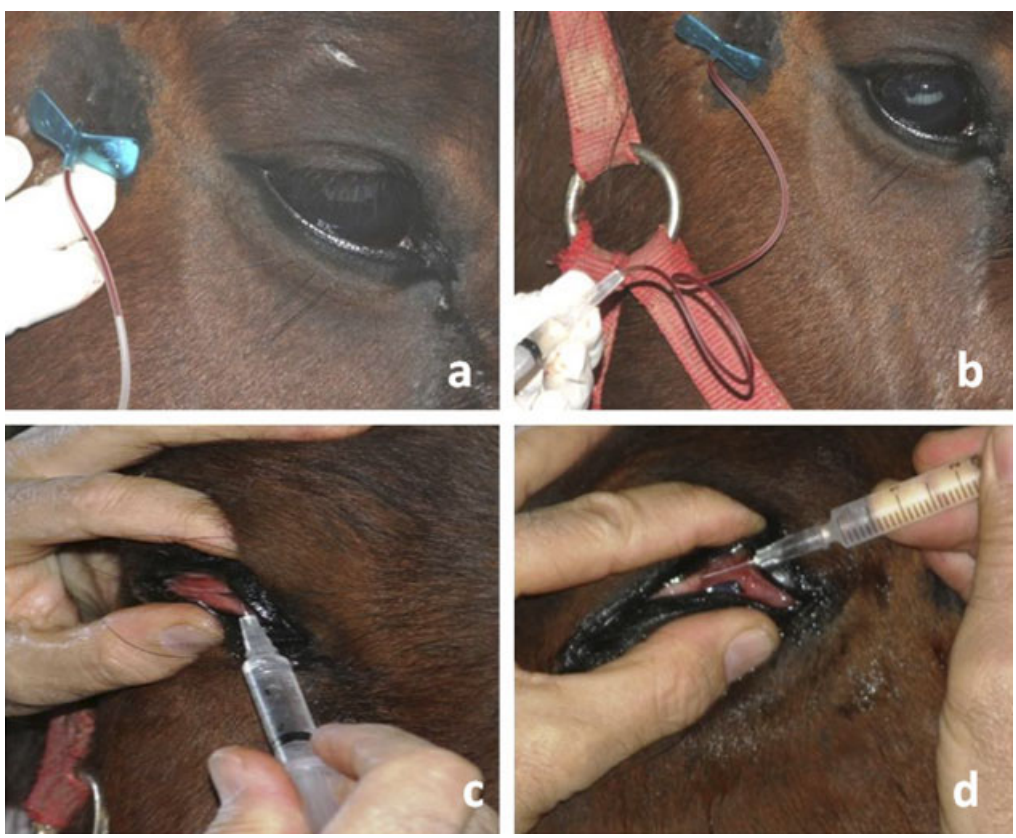

Fig. 1. Puncture (a) and injection (b) with a 21 G, 30 cm catheter of the artery providing the blood flow to the right (a, b) eye of a horse. Subconjunctival injection of the right (c) and left (d) eye of a horse.

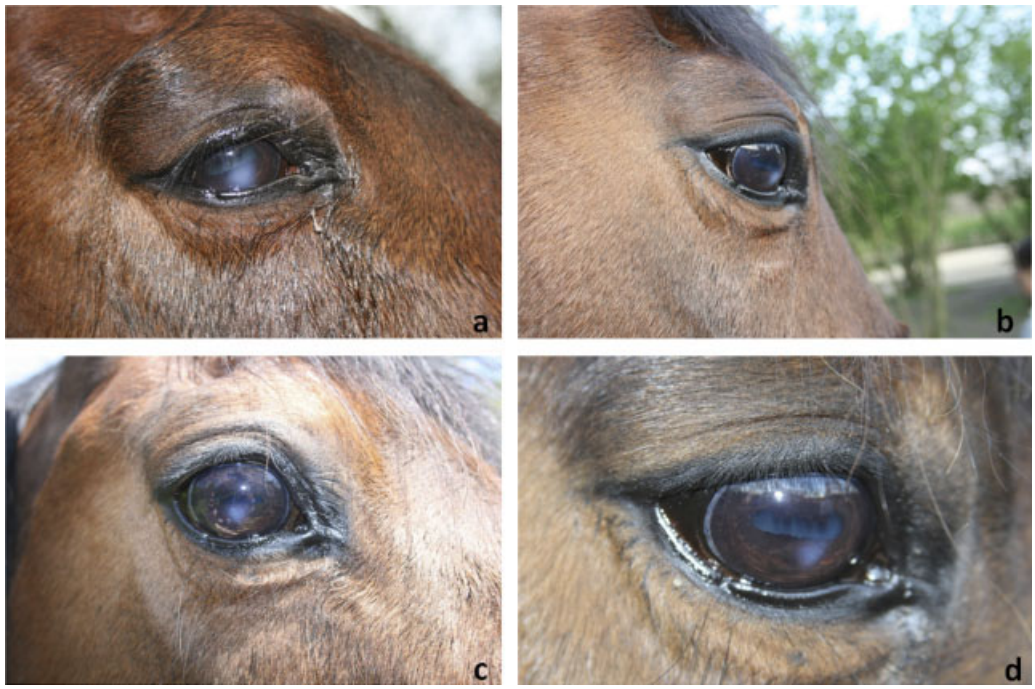

Fig. 2. Case 1: A 20-year-old horse with a bacterial ulcerative keratitis. Photos depicted visualize the inflamed and ulcerated right eye at the day of the stem cell therapy (a), after two weeks (b), and three months later (c, d).

further reduced and also the inflammation was stable without any signs of painfulness or irritation. Overall, the corneal ulcer disappeared after three months (Fig. 2c,d).

Case 2: This horse had been treated for a year with cycles of dexamethasone, tobramycin, and 1% atropine eye drops twice daily for two weeks. The regression of the symptoms lasted about three days and then reappeared more aggressively, making the treatment difficult (Fig. 3a). Also in this case, the horse was treated with administration of BDSCs IV and local instillation (as described above). After only two weeks, we observed an improvement (Fig. 3b) that was confirmed after one month without any signs of relapse (Fig. 3c).

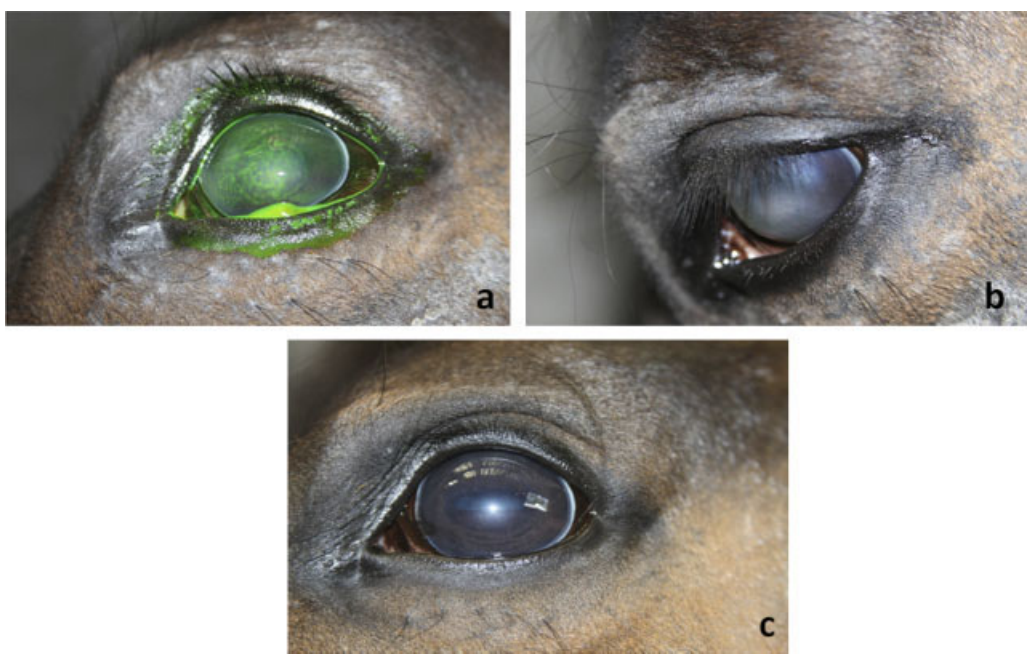

Fig. 3. Case 2: A 7-year-old German mare with an immune-mediated keratitis. Time 0 (a), after two weeks (b), and after one month (c).

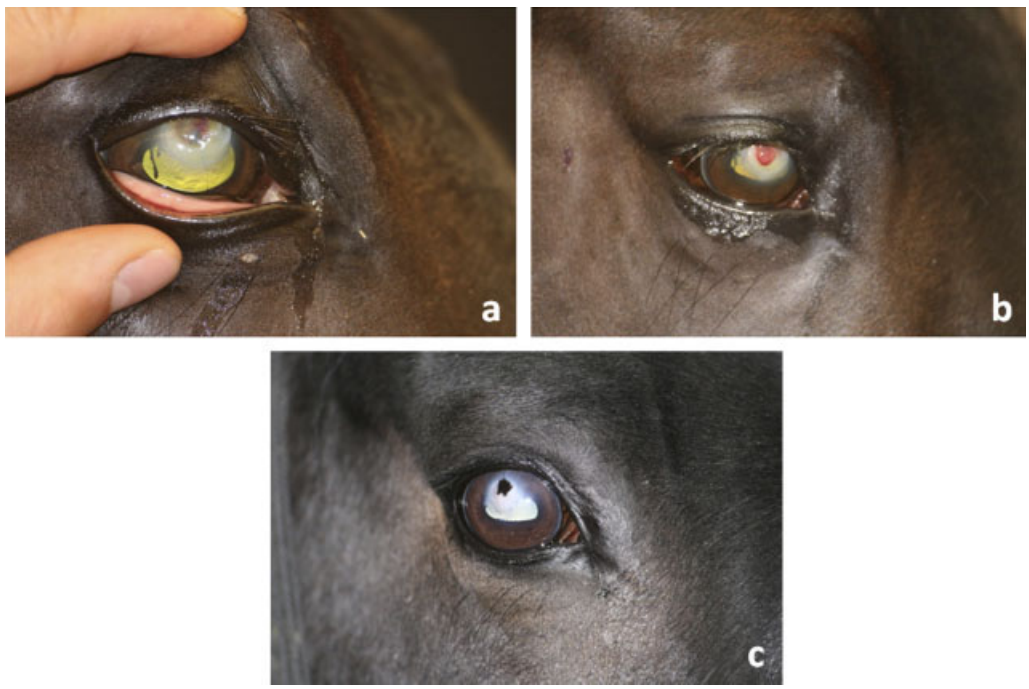

Fig. 4. Case 3: A 12-year-old gelding with a hornet sting on its cornea. Time 0 (a), after two weeks (b), and after one month (c).

Case 3: This case was treated with tobramycin and atropine 1% eye drops three times daily and also flunixin meglumine 0.5 mg/kg for two weeks. Since after six months the horse showed no improvement, it was treated with local and systemic stem cell therapy (as described above) (Fig. 4a). Two weeks later, the corneal ulcer was significantly reduced and the scarring effects were evaluated with a deposit of melanin after one month (Fig. 4b,c).

Case 4: The animal was treated daily with tobramycin and 1% atropine eye drops but the corneal ulcer was still evident after two weeks of treatment (Fig. 5a). Also in this case, the animal had undergone local and systemic stem cell treatment twice daily for two weeks (as described above). The corneal ulcer completely disappeared as shown by highlighting with fluorescein after two months (Fig. 5b,c,d).

Case 5: This young stallion presented with retinal detachment in both eyes and had been treated with systemic steroid and broad spectrum antibiotic therapy for two months, but the symptomatology remained unchanged (Fig. 6a). Therefore, it was the opinion of different ophthalmologists that the horse would not regain sight. In addition, this animal underwent administration of BDSCs IV. Three months later, the optic nerve regained function and visual acuity was improved in the left eye. After six months from the administration of BDSCs, the horse was able to jump and clear some cross-poles. The results were confirmed by ultrasound evaluation, which revealed much improvement in the left eye, where we observed a nearly complete "restitutio ad integrum," but did not reveal any noticeable improvement in the right eye (Fig. 6b). All cases are summarized in Table 2.

Discussion

Stem cells 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 in light of tendon and ligament injuries, has been described in different independent in vivo studies (Crovace et al., 2007; Schnabel et al., 2008; Smith, 2008). In order to be classified as a stem cell, it has to fulfill several requirements which are clearly defined for human stem cells (Dominici et al., 2006). Although no such strict definitions for veterinary stem cells have been established to date, it is generally accepted that MSCs, including those of equine origin, have to be positive for several typical MSC markers, and are able to differentiate into adipocytes, chondroblasts, and osteoblasts (Dominici et al., 2006). Equine MSCs are mostly characterized by the presence of CD73, CD90, and CD105, and the lack of CD14 and CD34 (Koerner et al., 2006; Vidal et al., 2006; Berg et al., 2009; Violini et al., 2009). Although MSCs hold great promise for future stem cell-based therapeutic strategies and indeed are currently employed clinically in equines,further research is required to understand their mechanisms of action in order to effectively dose and enhance these effects. There remains a huge knowledge gap in this field, as there is still a lack of precise definitions, characterizations, and standardization. Important essential knowledge is consequently lacking on how to tailor therapy.

In the present report, we describe the results obtained in four cases of corneal ulcer and one case of retinal detachment treated with administration of the animals' own BDSCs. After expansion and sorting, these stem cells were classified into three subpopulations: The first subpopulation was positive for two typical MSC markers, CD90 and CD105, but negative for CD73 (another MSC marker), indicating the commitment of peripheral blood stem cells towards the MSC lineage; the second one was positive for CD90, CD117, and CD 34, typical HSC markers, and the third one was positive for CD90, CD117 but also for Sox2, Oct3/4, and Nanog which are pluripotent stem cell markers. BDSCs can be a good source of stem cells since their use is characterized by low invasiveness, ease of collection and minimal pain to the animal (Zvaifler et al., 2000; Kassis et al., 2006). Furthermore, Koerner's study (2006)

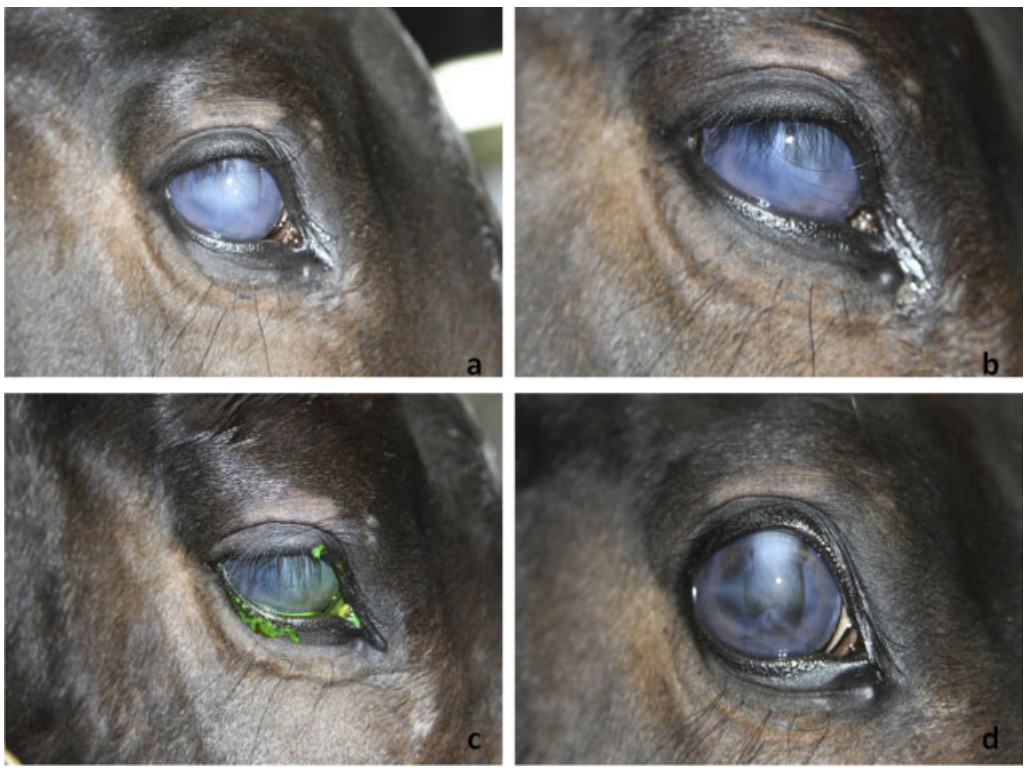

Fig. 5. Case 4: A 13-year-old gelding with a corneal ulcer as a complication from an ERU. Time 0 (a), after two weeks (b), after one month (c), and after two months (d).

showed that $CD34^{-}$ and $CD105^{+}$ progenitor cells obtained from ePB (equine peripheral blood) could clearly differentiate into osteocytes and adipocytes. In addition, the in vivo application of stem cells occurred without any side effects in all treated cases (Spaas et al., 2011).

Here, we individually describe the clinical characteristics of four animals with corneal ulcer and one with retinal detachment. In the first case study, peripheral blood stem cells were used to treat a horse with bacterial ulcerative keratitis. For six months, different therapies were tried without any success and, therefore, peripheral blood stem cells were used as a last resort. The second case was affected by immune-mediated keratitis with vascularization, edema, and an intense inflammatory response, and since the traditional therapies did not work here either, the animal was treated with stem cell therapy: Intravenously and locally instilled twice daily for two weeks. After three months, we observed a complete recovery from all symptoms, with no relapse within two months. The third case, a race horse, was traumatized by the sting of a hornet and subjected to daily antibiotic treatment for two weeks without any success. Also in this case, the ulcer disappeared after the stem cell therapy. The fourth case, affected by corneal

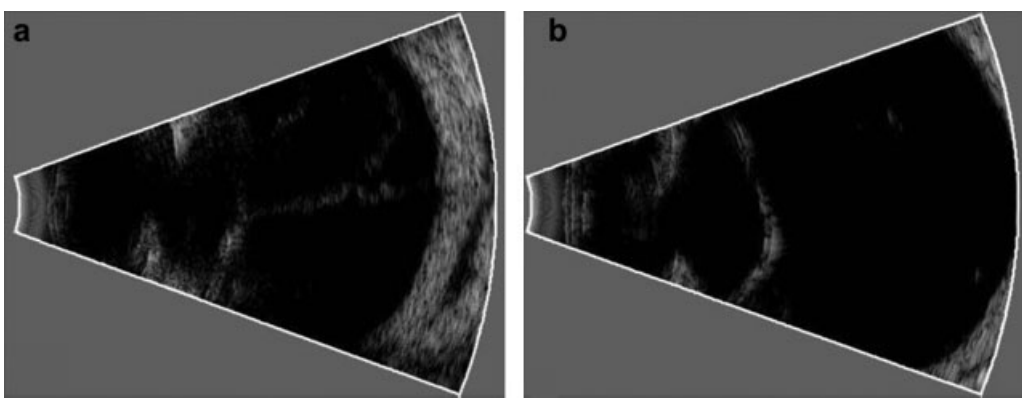

Fig. 6. Case 5: A 2-year-old stallion with retinal detachment. Ultrasound examination shows a retinal detachment at time 0 (a). Ultrasound examination shows the retina reattached after four months (b).

TABLE 2. Characteristics of five ophthalmological equine cases treated with BDSCs

Signalment	Affected region	Diagnosis	Treatment	Outcome
Case1				
20-year-old gelding	Cornea	Ulcer resistant to treatments	1 BDSCs IV and local instillation	Return to activity
Case 2				
7-year-old German mare (dressage)	Cornea	Immune-mediated keratitis	2 BDSCs IV and local instillation	Active
Case 3				
12-year-old gelding (jumping horse)	Cornea	Hornet sting	1 BDSCs IV and local instillation	Jumping
Case 4				
13-year-old gelding	Cornea	Uveitis	1 BDSCs IV	Return to activity
Case 5				
2-year-old stallion (jumping horse)	Retina and optic nerve	Retinal detachment	1 BDSCs IV	Active

ulcer as a complication from an ERU, was treated with stem cell therapy, which yielded good results after two weeks. The last case was a horse affected by bilateral retinal detachment (incomplete in the right eye, almost complete in the left eye). Three months after the administration of BDSCs, the optic nerve regained function and visual acuity at four months was improved in the left eye. Six months later, the horse was able to jump and clear some cross-poles.

The use of blood-derived stem cells as a possible treatment for corneal lesions or ulcers has not been described until now, although the use of eye stem cells and bone marrow-derived MSCs has been described previously as a treatment for corneal lesions in a large number of mammalian species, such as mice, rats, rabbits, horses, and humans (Shimazaki et al., 2002; Ueno et al., 2007; Girolamo, 2009; Koizumi et al., 2000; Jiang et al., 2010).

This study describes four different cases of ophthalmic pathology including a corneal ulcer and one case of retinal detachment that all show a positive outcome after peripheral blood stem cell therapy, but further scientific research is needed in order to understand their modus operandi and to propose a standardized protocol for using stem cell therapy in the treatment of human eye problems.

In conclusion, this report describes four cases of corneal ulcer and one case of retinal detachment in horses with a longstanding pathology, not responding to conventional treatment, where peripheral blood stem cells were used as a novel therapy. All horses showed significant improvement after such treatment, indicating the great potential of peripheral blood stem cell therapy. Still, many questions remain and more basic scientific research is certainly needed to fully understand the regenerative effects of stem cells in veterinary medicine in general, and the effects of these cells in equine regenerative therapy in particular.

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