Respiratory Physiology & Neurobiology 232 (2016) 35–42

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Intratracheal therapy with autologous bone marrow-derived mononuclear cells reduces airway inflammation in horses with recurrent airway obstruction

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ARTICLE INFO

Article history:
Received 14 April 2016
Received in revised form 10 June 2016
Accepted 7 July 2016
Available online 7 July 2016

Keywords:
Bronchoalveolar lavage
Heaves
Horses
Interleukins
Neutrophils
Pulmonary inflammation

ABSTRACT

This research evaluated the effects of bone marrow-derived mononuclear cells (BMMCs) on the inflammatory process in the equine recurrent airway obstruction (RAO). Eight horses in RAO clinical score were divided into cell therapy group (Gcel) treated with a single intratracheal dose of BMMCs, and dexamethasone group (Gdex) treated with 21 days of oral dexamethasone. The horses were clinically revaluated on days 7 and 21, together with cytological evaluation of the BALF, and detection of inflammatory markers (interleukins [IL]-10, -4, and -17, and interferon γ and α). There were decreases in respiratory effort and clinical score on days 7 and 21($p < 0.05$) for both groups. The percentage of neutrophils decreased and macrophages increased on days 7 and 21 ($p < 0.005$) in both groups. IL-10 levels increased in the Gcel group on day 21 compared to days 0 and 7 ($p < 0.05$), but this was not observed in the Gdex group. The quantification of IL-4, IL-17, IFN-γ, and IFN-α did not change between evaluations in both groups. These preliminary results suggest that BMMCs may ameliorate the inflammatory response of RAO.

1. Introduction

Horses are susceptible to chronic recurrent airway obstruction (RAO). Persistent airway inflammation develops in the presence of moldy hay, dusty straw, and pollens as a consequence of aberrant innate and adaptive immune responses, as well as genetic predisposition (Leclere et al., 2011). Maintaining horses in a pro-allergenic environment exacerbates the disease and causes significant airway neutrophilic influx, mucus accumulation, bronchospasm, bronchial hyper-reactivity, and airway obstruction and remodeling (Leclere et al., 2011; Pirie, 2014). Studies investigating the secretion of cytokines in horses with RAO horses showed contradictory results with regard to the Th1 and Th2 profiles (Pirie, 2014).

Horses with RAO exhibit increased respiratory effort at rest, coughing, and exercise intolerance, compromising athletic capacity and quality of life (Aharonson-Raz et al., 2012; Couëtil et al., 2016). Unfortunately, RAO has no cure, and conventional treatment with corticosteroids, although effective for controlling the inflammatory process and clinical signs, causes numerous adverse effects (Dauvillier et al., 2011). Therefore, there is an urgent need for new therapies.

The use of cell therapy has been investigated in a mouse model of asthma. Cell therapy was shown to control airway inflammation (Cho et al., 2015), with more pronounced benefits obtained with bone marrow-derived mononuclear cells (BMMCs) than with mesenchymal stem cells (MSCs) (Abreu et al., 2013).

RAO is an allergic disease that occurs naturally in horses. As there are similarities with human asthma with regard to the pathophysiology, RAO is considered to be an equine form of asthma, making

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http://dx.doi.org/10.1016/j.resp.2016.07.002
1569-9048/

the horse an ideal model for asthma studies (Leclere et al., 2011). However, cell therapy has not been studied in horses with RAO.

In the present study, the objective was to evaluate if the BMMCs therapy could ameliorate the clinical signs of horses with chronic RAO. We hypothesized that BMMCs would interfere with the response of cells involved in the inflammatory process, influencing the cellular profile and secretion of inflammatory cytokines in horses with RAO.

2. Materials and methods

2.1. Statement of animal care

This study was approved by the Committee on Animal Experimentation of the Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Brazil and is registered as number 788.

2.2. Animals

Eight horses (5 males and 3 females, mixed breed), weighing 480 ± 44.7 kg and aged 17.4 ± 6.11 years, with a history of chronic respiratory disease, episodes of dyspnea, and cough without symptoms of infection, were included in the study.

Prior to the initiation of the experimental protocol, the horses were dewormed and vaccinated against herpes virus and equine influenza, and they were maintained under pastured conditions for 3 months.

The horses were housed in stalls (2.5 × 2.5 × 4.0 m), bedded on sawdust that was overturned once a day, and fed moldy hay that was shaken near the nostrils for two minutes twice a day (Racine et al., 2011) until they exhibited clinical signs of RAO.

2.3. Study design

This was a randomized controlled experimental study to investigate the effects of BMMCs, compared with dexamethasone, in horses with RAO. As shown in Fig. 1, the eight animals were randomly divided into two groups: a cell therapy group (Gcel, n = 4) and a dexamethasone group (Gdex, n = 4).

Bone marrow was collected from the horses in the Gcel group during the three months spent on pasture, and the cells were cryopreserved in liquid nitrogen until the day of instillation. After confirmation of the diagnosis of RAO, horses in the Gcel group were sedated by intramuscular administration of acepromazine 1%[1] 0.05 mg/kg bwt (Acepran®) followed by intravenous administration of xylazine 10%[2] 0.5 mg/kg bwt (Sedomin®). A single infusion of 20 mL of autologous BMMCs that had been previously prepared was then instilled in the region of the carina with the aid of a 170 cm × 12 mm endoscope[3]. The volume contained between 5×10^8 to 1×10^9 cells and was thawed prior to instillation. Cell viability was confirmed by flow cytometry using Anexin-V[4] and 7-aminoactinomycin[4] (7-AAD). After the procedure, each animal's head was gently held in an elevated position for 10 min to prevent the return of the instilled cells.

The Gdex group was treated with oral dexamethasone at decreasing doses: 0.165 mg/kg (days 0–7), 0.083 mg/kg (days 8–14), and 0.04 mg/kg (days 15–2) (DeLuca et al., 2008).

After the onset of treatment, the induction was stopped, but the horses were kept stabled on sawdust throughout the study period. Evaluations were performed 7 and 21 days after treatment was initiated (Leclere et al., 2011; Cruz et al., 2012).

2.4. Bone marrow collection

Horses were sedated by intramuscular administration of acepromazine 1%[1] 0.05 mg/kg bwt (Acepran®). Then, the collection site between the fourth and sixth sternebrae (Alves et al., 2009; Kasashima et al., 2011) was trichotomized, aseptically prepared, and locally anesthetized with lidocaine 2%[5] (Lidovet®). Sedation was complemented with intravenous administration of xylazine 10%[2] 0.5 mg/kg bwt (Sedomin®) together with 50 mg of intravenous pethidine hydrochloride[6] (Dolosal®). Upon insertion of a 11 G × 10 cm Jamshidi needle, 200 mL samples were collected from each animal at two to four sites by aspiration into sterile 20 mL syringes containing 7 mL of IMDM[7] (Iscove's Modified Dulbecco's Medium) and 0.5% of sodium heparin 5000 IU/mL[6] (Hemofol®). The syringes were kept on ice during transport to the laboratory and were processed within 2 h of sample collection.

2.5. Bone marrow processing

Aliquots of 300 μL of the samples were filtered through a 100 μm mesh[4] (cell strainer) and separated to determine the total nucleated cell count (TNCC) using an automatic hematology analyzer[8] (2800 BCE). After counting, the samples were processed according to the technique of Boyum adapted (Boyum, 1964), using the density gradient[7] (Ficoll/Histopaque®-1077) to obtain the BMMCs. After performing another TNCC, the material was prepared for cryopreservation in an aqueous solution containing 10% fetal bovine serum[9], 10% dimethylsulfoxide[7] (DMSO), and BMMCs in IMDM. The cells were stored in appropriate containers at −80 °C overnight and then transferred to liquid nitrogen at −196 °C until the time of instillation.

2.6. BMMCs preparation

For cell therapy, BMMCs were thawed quickly in a water bath at 37 °C, diluted in IMDM with fetal bovine serum, and centrifuged at 400*g* for 10 min. The pellet was resuspended in 15 mL of a 0.9% sodium chloride solution and filtered through a 100 μm mesh[4] (cell strainer). The number of cells was determined using an automatic hematology analyzer[8] (2800 BCE). An aliquot of cells was reserved for flow cytometry to verify viability using the reagents Anexin V[4] and 7-AAD[4]. Finally, 5×10^8 to 1×10^9 BMMCs were added to 5 mL of fresh autologous serum to prepare a 20 mL sample for instillation.

2.7. Physical exam

Physical exam and blood analysis were performed before the study, while animals were living on pasture, to exclude other medical disorders. All horses were examined in the morning (Hoffman, 2008). The nostrils, lymph nodes, cough reflex, respiratory rate, lung sounds, heart rate, and rectal temperature were examined (McGorum et al., 2007). Respiratory effort was determined by the presence of the heave line, and the severity was graded as 3–strong, 1–light, and 0–absent (Tesarowski et al., 1996). Endoscopy was performed (Gerber et al., 2004) and bronchoalveolar lavage fluid (BALF) was collected (Aharonson-Raz et al., 2012). The clinical condition was determined for each animal, and the information was then used to determine a clinical score, as suggested by Tesarowski et al. (1996). The horses were diagnosed with RAO on the basis of their clinical history, a clinical score ≥10 points, and BALF cytology (≥25% neutrophils) after exposure to moldy hay and dusty bedding.

2.8. BALF collection and analysis

BALF collection was performed after sedating the animalsby intravenous administration of detomidine[10] 0.005 mg/kg bwt (Eqdomin®) and 50 mg of intravenous pethidine hydrochloride[6] (Dolosal®). A 3 m × 1 mm silicon catheter[11] (V-300 PBAL) was introduced intranasally, and four 125 mL aliquots of sterile warm (37 °C) 0.9% sodium chloride solution (Hoffman, 2008) were infused and