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

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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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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

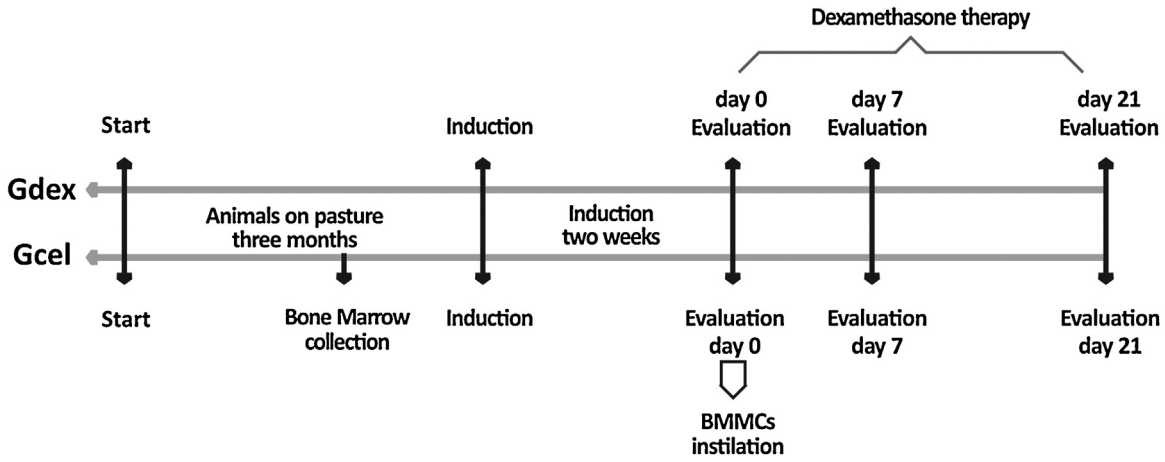

Fig. 1. Scheme of the experimental design for the eight animals in the study, from the beginning of the experiment until the last evaluation, 21 days after the start the treatment of horses with recurrent airway obstruction.

immediately retrieved. In the laboratory, the BALF was centrifuged (350*g* at 4 °C for 6 min), and the cell pellet was resuspended in 2 mL of the BALF supernatant from the same animal for the TNCC. Cell counting was performed using a Neubauer chamber, and differential cytological analysis was performed using slides stained with Diff-Quick[12] (Panótico Rápido) and counting 300 cells under 1000× magnification.

2.9. Interleukin analysis

The BALF supernatant from each collection was aliquoted and stored at −20 °C for the analysis of IL-4, IL-10, IL-17, interferon (IFN)-γ, and IFN-α by flow cytometry with the 5-plex assay[13].

2.10. Statistical analysis

The statistical analysis included comparisons between groups and comparisons between evaluation times within each group; the results at days 7 and 21 were compared with the initial evaluation for all of the variables investigated. The data were analyzed using Graphpad Prism 5.0 software. Initially, the normality of the distribution of the data was evaluated by the D'Agostino and Pearson test. For qualitative analysis (respiratory effort and clinical score), the Wilcoxon test was used. For quantitative analysis (neutrophils, macrophages, and interleukins), repeated measures ANOVA followed by the Tukey test was used. The results are shown as the mean ± standard deviation (SD). Differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Animals

Twelve horses were planned to be included in the study. However, two horses did not develop a sufficiently high clinical score for inclusion, and two horses enrolled in the Gcel group not provide the number of BMMCs expected for instillation. All of these horses were removed from the study. Therefore, the study was conducted with the eight animals that developed the clinical signs of RAO and reached the clinical score of at least 10.

3.2. Bone marrow collection and processing

None of the horses exhibited discomfort or any clinical condition after undergoing the bone marrow collection procedure.

The average instillation of BMMCs was $972.2 \pm 313.9 \times 10^6$ cells, with a cell viability of 93.2 ± 3.8% after thawing (Table 2), which was accordance with the proposal of the study.

3.3. Physical exam

In this study, abdominal effort was visible in all animals based on the presence of a heave line before the treatments were initiated (Table 1). After treatment, there was a significant decrease in the respiratory effort at day 7 in the Gdex group ($p = 0.030$) and at day 21 in both groups ($p = 0.012$ for Gdex, and $p = 0.030$ for Gcel) compared with day 0 (Fig. 2A). The clinical score also decreased significantly at day 7 ($p = 0.013$ for Gdex, and $p = 0.014$ for Gcel) and at day 21 ($p = 0.014$ for Gdex, and $p = 0.014$ for Gcel) compared with day 0 (Fig. 2B). The clinical score and the abdominal effort had no differences observed between groups.

3.4. BALF collection and analysis

The data obtained from BALF collection in the Gdex and Gcel groups are shown in Table 1.

Changes were observed in the BALF cell profiles in both groups, with a significant decrease in the percentage of neutrophils (Fig. 3A) at day 7 vs. day 0 ($p < 0.0001$ for Gdex, and $p = 0.0028$ for Gcel) and at day 21 vs. day 0 ($p < 0.0001$ for Gdex, and $p = 0.0024$ for Gcel). No differences were observed between groups.

This decrease in the percentage of neutrophils was accompanied by a significant increase in the percentage of macrophages (Fig. 3B) at day 7 vs. day 0 ($p < 0.0001$ for Gdex, and $p = 0.0010$ for Gcel) and at day 21 vs. day 0 ($p < 0.0001$ for Gdex, and $p = 0.0023$ for Gcel). No differences were observed between groups.

3.5. Interleukin analysis

The level of IL-10 in the BALF of horses in the Gcel group was higher at 21 days of treatment than at days 0 and 7 (111.3 ± 107.1 vs. 22.8 ± 14.6 and 15.8 ± 4.9 pg/mL, $p = 0.036$ and $p = 0.034$, respectively). There was no change in IL-10 in the Gdex group (day 0 = 6.0 ± 12.0, day 7 = 19.8 ± 39.5 and day 21 = 4.3 ± 7.8 pg/mL) (Fig. 4).

The quantification of IL-4, IL-17, IFN-γ, and IFN-α did not change between evaluations in both groups.

Table 1
Individual percentage of recovered volume, total nucleated cell count, percentage of different cell types in bronchoalveolar lavage fluid and clinical score of horses with Recurrent Airway Obstruction of the group treated with dexamethasone (Gdex) and the group treated with tracheal instillation of bone marrow-derived mononuclear cells (Gcel), for each evaluation.

	BALF Volume%	TNCC (cells/μL)	Neutrophils %	Macrophages %	Lynphocytes %	Respiratory rate (0–4)	Nasal discharge (0–3)	Abdominal lift (0–3)	Nasal flaring (0–1)	Tracheal sounds (0–3)	Bronchial tones (0–3)	Crackles (0–2)	Wheezes (0–2)	Cough (0–3)	Total Clinical Score
Dex 1 0d	42.4	116.51	46.0	40.0	10.0	1	1	1	0	0	2	2	0	3	10
Dex 1 7d	53.0	156.23	7.0	70.0	17.0	0	1	0	0	0	2	2	0	0	5
Dex 1 21d	42.6	89.67	2.0	85.0	3.0	2	0	0	0	1	0	2	0	0	5
Dex 2 0d	40.0	37.45	67.0	23.0	7.0	3	1	1	0	1	2	2	0	1	11
Dex 2 7d	56.0	206.07	19.0	66.0	12.0	1	1	1	0	0	2	2	0	0	7
Dex 2 21d	39.2	85.71	21.0	49.0	19.0	1	3	0	0	1	0	2	0	1	8
Dex 3 0d	50.8	74.80	60.0	22.0	17.0	0	1	3	0	3	2	2	0	1	12
Dex 3 7d	63.8	79.00	13.0	55.0	20.0	0	1	1	0	3	0	2	0	0	7
Dex 3 21d	49.4	53.24	11.0	70.0	16.0	0	1	1	0	0	2	2	0	0	6
Dex 4 0d	59.4	77.44	79.0	12.0	8.0	0	3	3	1	3	2	2	0	3	17
Dex 4 7d	68.2	61.58	24.0	54.0	20.0	1	2	1	0	1	0	2	0	1	8
Dex 4 21d	66.2	100.00	26.0	36.0	32.0	0	1	1	0	1	0	2	0	0	5
Cell 1 0d	49.0	21.63	85.0	29.0	43.0	0	1	1	1	1	2	2	0	3	11
Cell 1 7d	55.0	61.45	23.0	62.0	12.0	0	1	1	0	1	2	2	0	1	8
Cell 1 21d	51.4	88.33	8.0	47.0	40.0	0	1	1	0	0	2	2	0	1	7
Cell 2 0d	42.4	130.66	49.0	30.0	13.0	1	3	1	1	3	2	2	0	0	13
Cell 2 7d	49.8	65.46	1.0	46.0	52.0	0	1	0	0	0	2	2	0	0	5
Cell 2 21d	37.4	48.66	9.0	67.0	20.0	0	1	0	0	0	0	2	0	0	3
Cell 3 0d	36.1	405.38	70.0	17.0	3.0	1	1	3	1	1	2	2	2	3	16
Cell 3 7d	36.2	113.81	48.0	29.0	15.0	0	1	1	1	3	2	2	0	1	11
Cell 3 21d	50.0	76.00	68.0	26.0	3.0	0	1	1	1	0	2	2	0	1	8
Cell 4 0d	69.4	24.21	36.0	28.0	15.0	1	3	1	1	1	2	2	0	1	12
Cell 4 7d	73.4	65.94	4.0	72.0	23.0	0	1	1	0	0	2	2	0	0	6
Cell 4 21d	67.8	108.65	3.0	79.0	15.0	0	1	0	0	0	2	2	0	0	5

BALF = bronchoalveolar lavage fluid; TNCC = total nucleated cell count; Dex = Gdex group; Cell = Gcel group.

Table 2
Individual data for bone marrow collection in adult horses and processing of bone marrow-derived mononuclear cells.

	BM volume	BMMCs pre-isolation	BMMCs post-isolation	BMMCs instilled	Viability
	mL	Billions	Billions	millions	%
H1	226.50	9.45	1.09	651.50	92.72
H2	226.50	19.90	2.19	811.36	88.00
H3	281.50	22.00	1.08	826.00	98.00
H4	198.00	39.00	3.70	1600.00	94.24
mean ± SD	233.13 ± 34.94	22.59 ± 12.24	2.02 ± 1.24	972.22 ± 425.92	93.24 ± 4.14

BM = bone marrow; BMMCs = bone marrow-derived mononuclear cells; H = horse; SD = standard deviation.

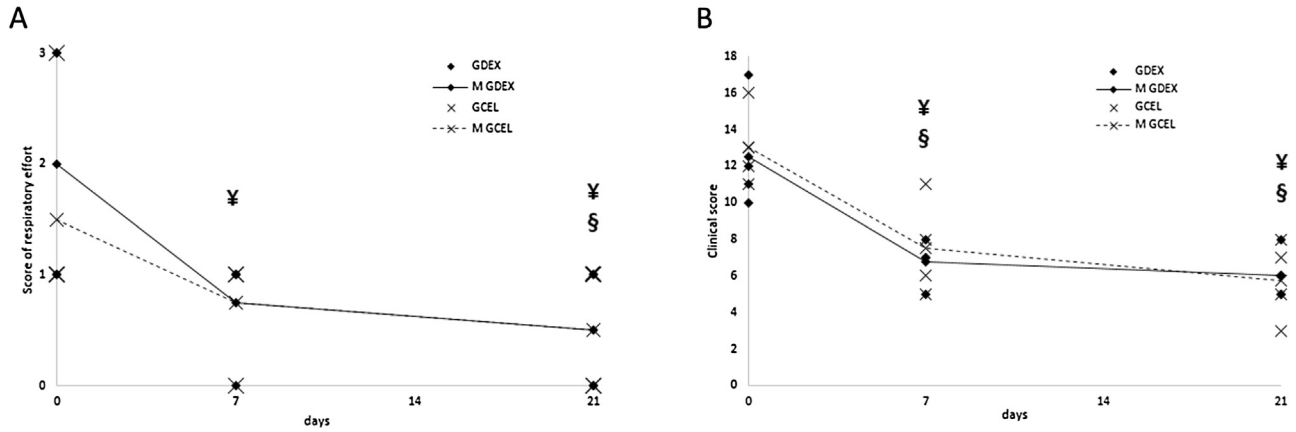

Fig. 2. (A) Score for respiratory effort (0–3 – absent to strong) in horses with recurrent airway obstruction treated with oral dexamethasone (Gdex) or tracheal instillation of autologous bone marrow-derived mononuclear cells (Gcel). (B) Clinical scores of horses with recurrent airway obstruction (≥10 points – RAO-affected horses) treated with oral dexamethasone (Gdex) or tracheal instillation of autologous bone marrow-derived mononuclear cells (Gcel). ¥ $p < 0.05$ compared with Gdex day 0; § $p < 0.05$ compared with Gcel day 0.

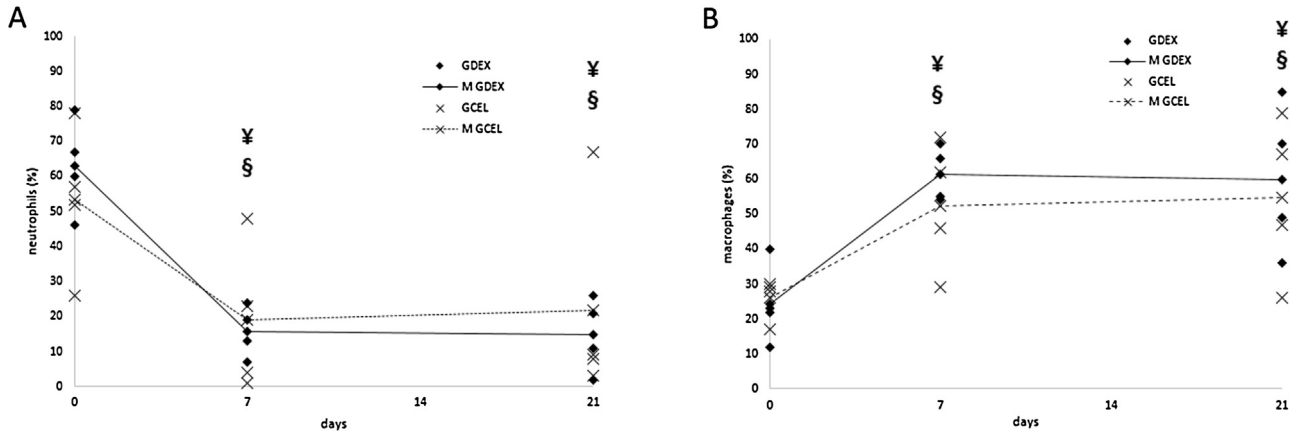

Fig. 3. (A) Percentage of neutrophils in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction (≥25% – RAO affected horses) treated with oral dexamethasone (Gdex) or tracheal instillation of autologous bone marrow-derived mononuclear cells (Gcel). (B) The percentage of alveolar macrophages in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction (50–70% – healthy horses) treated with oral dexamethasone (Gdex) or tracheal instillation of autologous bone marrow-derived mononuclear cells (Gcel). ¥ $p < 0.05$ compared with Gdex day 0; § $p < 0.05$ compared with Gcel day 0.

4. Discussion

RAO has been widely studied in veterinary medicine, but we are not aware of another study using cell therapy, despite promising results in investigations using MSCs and BMMCs in asthma models (Abreu et al., 2013; Cruz et al., 2012). Thus, in the present study, consistent with the observations in mice (Firinci et al., 2011), the use of intratracheally instilled BMMCs had beneficial effects on the clinical signs and immune response in horses with RAO. In this study, we treated all horses in the control group with conventional therapy with dexamethasone (DeLuca et al., 2008).

Cell therapy can be performed using several types of cells. BMMCs have shown good results in a previous study with a TH-2-mediated eosinophilic mouse model of asthma (Abreu et al., 2013). On the basis of this information, we used BMMCs for this preliminary study regarding cell therapy in horses with RAO, even though RAO is characterized by an increase in BALF neutrophils. However, a recent study demonstrated that the BMMCs were also effective in the treatment of a mixed TH-2/TH-17-mediated neutrophilic airway inflammation in mice, showing an improvement in the BALF inflammatory cells (Cruz et al., 2016), similar to the observations of our study. In addition, BMMCs have several advantages over MSCs. BMMCs result in less lung remodeling and can be isolated using

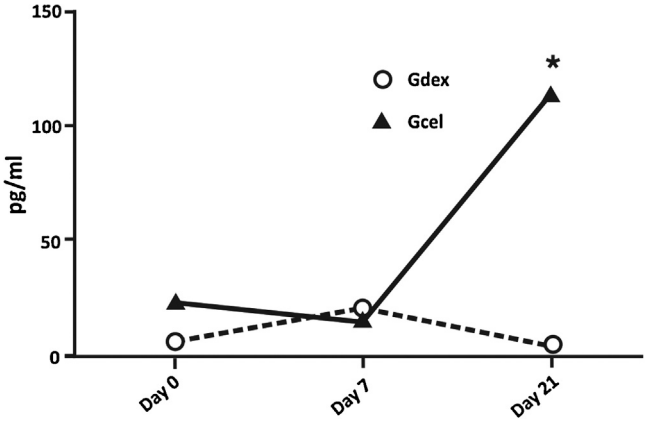

Fig. 4. Measurement of interleukin-10 in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction treated with oral dexamethasone (Gdex) or tracheal instillation of autologous bone marrow-derived mononuclear cells (Gcel). *$p < 0.05$ compared with day 0 and day 7.

a simple protocol, with lower costs and shorter processing times. Moreover, cell therapy with BMMCs can be applied on the day of bone marrow collection (Abreu et al., 2013; Cruz et al., 2016).

In this study, the BMMCs were isolated for cryopreservation during the period when the horses were kept on pasture and were in a stable respiratory condition. Cryopreservation was performed because several studies have found that the activity of the BMMCs is reduced in patients with certain health issues (Heeschen et al., 2004); in asthmatic patients, decreased cell numbers and reduced cellular activity may have been caused by impaired mobilization of progenitor cells from the bone marrow (Denburg et al., 2000). There is a debate regarding the freshly thawed cell activity in this procedure and its viability as a therapy. A recent study demonstrated that freshly thawed cells were as effective as continuously cultured cells in ameliorating lung inflammation in a mouse model of allergic airway inflammation (Cruz et al., 2015), which led us to choose to not submit elderly and sick animals to the collection of bone marrow.

The number of BMMCs used (5×10^8 to 1×10^9) was established based on extrapolations of cell therapies performed in other species and other equine diseases, as we are not aware of studies on RAO or any other respiratory disease of horses. Cell therapy for equine tendons requires an average dose of 1×10^7 to 2×10^7 million cells (Godwin et al., 2012; Schnabel et al., 2013), and in ischemic diseases in humans, the number of cells used is approximately 1.7×10^9 billion (Murphy et al., 2011).

The intratracheal route of administration was chosen because, in horses, this route is easily accessed through a routine endoscopic procedure. Moreover, the intratracheal route ensures the delivery of a higher number of cells directly into the airways (Bonios et al., 2011).

The cell viability obtained after thawing the cells was highly satisfactory, approaching $93.2 \pm 3.8\%$ recovery, compared with a study by Ribeiro et al. (2012), who reported 86% viability. In the equine species, little is known about the effects of cryopreservation on cell viability or about the relationship between the protocols used and cell damage caused by the formation of ice crystals (Sumida, 2006).

Considering the clinical signs of RAO in the affected horses, increased breathing effort during rest is an important clinical component indicating impaired pulmonary gas change. Therefore, in the present study we meticulously determined the clinical score of the investigated animals and assumed that horses with clinical scores ≥ 10 exhibited pulmonary dysfunction. It was previously demonstrated that RAO-affected horses with a clinical score ≥ 6 had a significant increase in $PaCO_2$ and a decrease in PaO_2 (Tesarowski et al., 1996). The clinical score decreased significantly after both treatments, to ≤ 6 on an average, indicating that the animals had significant improvement in the quality of respiratory function due to both the dexamethasone and BMMCs treatments.

For healthy horses, neutrophils account for 5% of the cells in BALF, and macrophages represent 50–70% (Hoffman, 2008). The decrease in the number of neutrophils and the increase in the number of macrophages in BALF was consistent with previous observations in horses treated with oral dexamethasone (Robinson et al., 2002), even when the animals were kept in the stable (Rush et al., 1998). The cell therapy performed in the present study resulted in the same level of improvement as that seen with the conventional therapy, with the advantage of being a monotherapy with a reduced risk of side effects in addition to being an autologous therapy. However, although potent immunomodulatory effects produced by treatments with BMMCs have been described, we still know little about their mechanisms of action (Schnabel et al., 2013).

The significant decrease in neutrophils in BALF was demonstrated by other authors by using cell therapy with both BMMCs and MSCs, which are a part of the mononuclear pool, for treatment in a murine model of asthma (Lathrop et al., 2014; Braza et al., 2016; Cruz et al., 2016). The BMMCs are a heterogeneous pool of cells, possessing fractions of hematopoietic and mesenchymal cells, endothelial progenitor cells (Murphy et al., 2011), lymphocytes, and monocytes. Taking into account that many studies have reported impressive results by using only the MSCs, it is commonly believed that the mesenchymal fraction is uniquely responsible for the beneficial effects of BMMCs. In a recent study, Cruz et al. (2016) used the depletion of different cell fractions present in BMMCs, demonstrating that the removal of the monocyte fraction, as well as the MSC, inhibited improvement in pulmonary inflammation in a mouse model of severe asthma, proving that the effect of BMMCs is attributed not only to MSCs but also to an interaction amongst the cell populations.

Another important result of this study was the increase in interleukin (IL)-10 in the group treated with cell therapy, which was significantly higher than that in the group treated with dexamethasone. IL-10 is known to inhibit the generation of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8, and it is a potent inhibitor of macrophage function (Chung and Barnes, 1999; Ya-xia, 2012). In our previous study, the adhesion and phagocytosis activities of the alveolar macrophages in the BALF were not affected in the Gcel group, which had increased IL-10 level; however, the Gdex group showed significant increases in both adhesion and phagocytosis (Bastos, 2015) (data not shown). This corroborates the findings of other authors showing that IL-10 in an inhibitor of macrophage activity (Chung and Barnes, 1999), in addition to its role in the polarization of M2 macrophages, which are important for immunoregulation (Mantovani et al., 2013).

Asthmatic humans have lower levels of IL-10 than healthy humans do, and the *in vitro* treatment with corticosteroids induces a reduction in the release of IL-10 by macrophages (John et al., 1998). In contrast, intratracheal instillation of cell therapy in asthmatic mice resulted in an increase in IL-10 level in the BALF (Ge et al., 2013). These findings are similar to the results of our study, where treatment with corticosteroids maintained IL-10 at a low level in the Gdex group, but IL-10 level increased in the Gcel group. Again, the immunomodulatory effects of BMMCs may arise in diseases with major involvement of chemical mediators. Several studies have shown that high levels of IL-10 can induce long-lasting tolerance and can substantially reverse the asthma phenotype, but the effect is not permanent (Nayyar et al., 2012). Moreover, IL-10 can suppress airway hyper-responsiveness and can block smooth muscle cell activation by inflammatory stimuli, preventing airway remodeling (Mazighi et al., 2004).

Besides similarities between the results obtained with both therapies, the difference between them is that conventional therapy only causes regression of the inflammatory process, while cell therapy may protect or even repair the site of disease and may improve the tolerance to allergens. RAO tends to affect mature-to-older horses (Couëtil et al., 2016) or horses aged ≥7 years (Aharonson-Raz et al., 2012)that have had contact for a sufficient time with the atmosphere of the stables and have been fed hay (Robinson et al., 2002). The animals in our study were very old, with an average age of 17.4 ± 6.11 years, and the results may be even better in animals experiencing the first episodes of RAO.

5. Conclusion

In the present study, treatment of RAO in horses with an intratracheal instillation of BMMCs was effective in improving the clinical signs, and safe. This treatment had different immunomodulatory potential compared with conventional therapy, increasing the levels of the anti-inflammatory cytokine IL-10. Additional studies are needed to understand the effects of BMMCs on disease regression as well as the mechanisms that underlie these effects.

Manufacturers' details

[1]Univet Laboratory, São Paulo, Brazil.
[2]König, Buenos Aires, Argentina.
[3]Olympus, São Paulo, Brazil.
[4]BD Biosciences, New Jersey, USA.
[5]Bravet Laboratory, Rio de Janeiro, Brazil.
[6]Cristália Laboratory, Itapira, Brazil.
[7]Sigma-Aldrich, Missouri, USA.
[8]Mindray, Shenzhen, China.
[9]Gibco, Invitrogen, South America.
[10]Ouro Fino Laboratory, Cravinhos, Brazil.
[11]Cook Vet Products, Indiana, USA.
[12]Laborclin, Pinhais, Brazil.
[13]Animal Health Diagnostic Center in Cornell University, New York, USA.

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Conflict of interest

Authors disclose no conflict of interest.

Authorship

Fernanda Zettel Bastos and Fernanda Cristina Mendes Barussi participated in study design, experiments accomplishment (horse exam, airway endoscopy, bronchoalveolar lavage obtainment and processing, bone marrow obtainment and processing), data analysis, manuscript preparation; Pedro Vicente Michelotto Júnior participated in study design, helped in experiments (horse exam, airway endoscopy, bronchoalveolar lavage obtainment and processing), data analysis, manuscript preparation; Cláudia Turra Pimpão participated in study design, data statistical analysis, final manuscript approval; Anita Nishiyama participated in study design, analysis of alveolar macrophages and in the final manuscript approval; Lidiane Maria Boldrini Leite, Felipe Yukio Ishikawa Fragoso, Alexandra Cristina Senegaglia and Paulo Roberto Slud Brofman participated in study design, gave support on cell therapy throughout the study period including the training for bone marrow processing, data analysis, final manuscript approval.

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