

Insights into animal models for cell-based therapies in translational studies of lung diseases: Is the horse with naturally occurring asthma the right choice?

ANNA LANGE-CONSIGLIO^{1,4}, LUCA STUCCHI², ENRICA ZUCCA²,
 JEAN PIERRE LAVOIE³, FAUSTO CREMONESI^{1,4} & FRANCESCO FERRUCCI²

¹Department of Veterinary Medicine, Università degli Studi di Milano, Milano, Italy, ²Equine Medicine Unit, Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Milano, Italy, ³Department of Clinical Sciences, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada, and ⁴Reproduction Unit, Centro Clinico-Veterinario e Zootecnico Sperimentale di Ateneo, Università degli Studi di Milano, Lodi, Italy

Abstract

Human asthma is a widespread disease associated with chronic inflammation of the airways, leading to loss of quality of life, disability and death. Corticosteroid administration is the mainstream treatment for asthmatic patients. Corticosteroids reduce airway obstruction and improve quality of life, although symptoms persist despite treatment in many patients. Moreover, available therapies failed to reverse the lung pathology present in asthma. Animal models, mostly rats and mice, in which the disease is experimentally induced, have been studied to identify new therapeutic targets for human asthma. Alternative animal models could include horses in which naturally occurring asthma could represent an important step to test therapies, potentially designed around mouse studies, before being translated to human testing. Horses naturally suffer from asthma, which has striking parallels with human asthma. Severe equine asthma (SEA) is characterized by reversible bronchospasms and neutrophil accumulation in the lungs immunologically mediated mainly by Th2. Moreover, the pulmonary remodelling that occurs in SEA closely resembles that of human asthma, making the equine model unique for investigation of tissue repair and new therapies. Cell therapy, consisting on mesenchymal stromal cells (MSCs) and derivatives (conditioned medium and extracellular vesicles), could represent a novel therapeutic contribution for tissue regeneration. Cell therapy may prove advantageous over conventional therapy in that it may repair or regenerate the site of injury and reduce the reaction to allergens, rather than simply modulating the inflammatory process.

Key words: equine, extracellular vesicles, mesenchymal stromal cells, regenerative medicine, respiratory disease

Introduction

Human asthma is a chronic disease and consists of airway inflammation and remodelling that may contribute to hyper-responsiveness and chronic airway obstruction [1]. It has been estimated that asthma afflicts more than 300 million people, with a prevalence ranging from 1–18% in various countries, and it is the most common chronic disease in childhood [2]. The pathological features of airway remodelling include fibrosis of the sub-epithelium, hypertrophy and hyperplasia of the airway smooth muscle and hyperplasia and hypersecretion of the mucous glands (Figure 1A, 1B and 1C) [3]. In conjunction with airway remodelling, the production of inflammatory mediators, growth factors, cytokines,

extracellular matrix proteins and reactive oxygen species is increased [4,5]. This airway remodelling can cause irreversible airway obstruction, which can contribute to worsening the severity of asthma. [5].

Asthma and conventional therapies

Human asthma, although mainly characterized by an eosinophilic inflammation of the lung, can be considered a heterogeneous disease. Several phenotypes of asthma have been defined, with a significant number of patients suffering from a non-eosinophilic form of asthma [6]. Treatments of asthma aim to achieve symptoms control and to prevent future exacerbations.

Correspondence: Anna Lange-Consiglio, PhD, Department of Veterinary Medicine, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy.
 E-mail: anna.langeconsiglio@unimi.it

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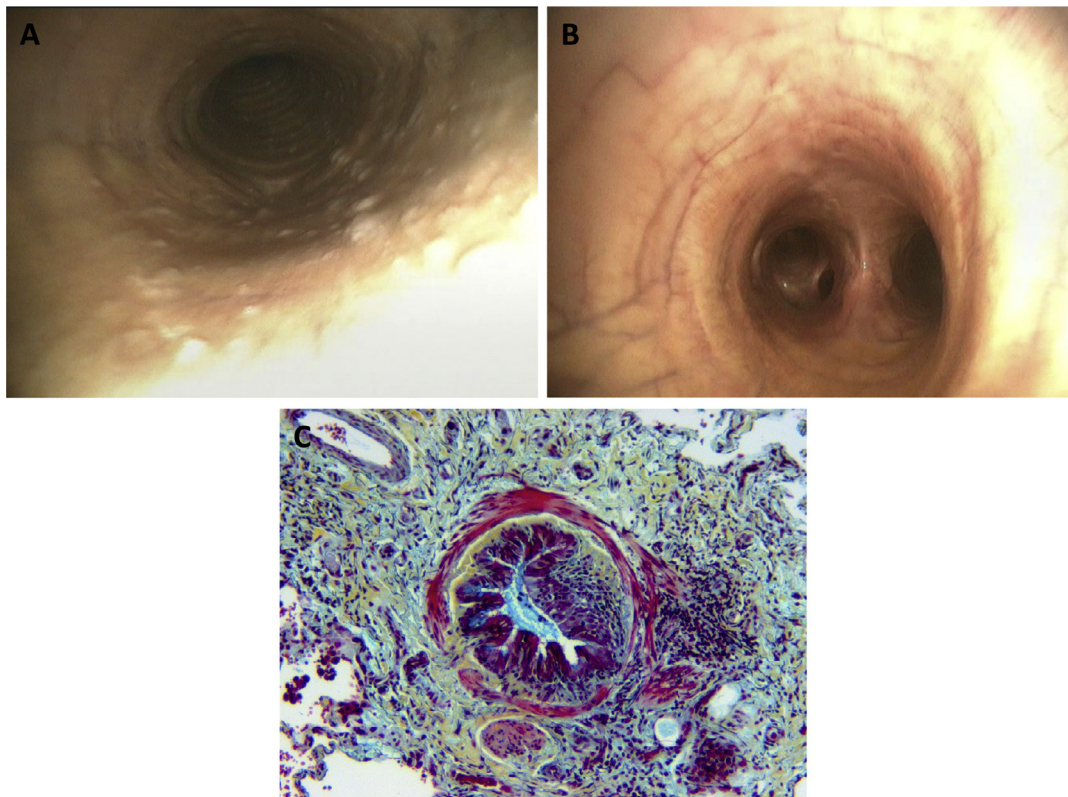


Figure 1. Pathological features of asthma. (A) Tracheal mucus accumulation in a horse with asthma. (B) Endoscopic view of inter-bronchial carena in an asthmatic horse. Note the edema of the tissues. (C) Peripheral lung tissues from a horse with severe asthma. Alterations affecting the small airways include mucus and inflammatory cell accumulations with the airway lumen, hyperplasia of mucus cells (blue), deposition of collagen (yellow) in the lamina propria and thickening of the airway smooth muscle layer (red). Movat pentachrome stain.

Pharmacological therapies include mainly bronchodilators (i.e., β_2 -agonists) to address airflow limitations and inhaled corticosteroids (such as beclomethasone or fluticasone) to limit airway inflammation, which are modulated based on disease severity. Nevertheless, patients with severe asthma are incompletely controlled with these treatments and recurrent exacerbations may occur despite long-term therapy [7]. Treatment resistance to asthmatic attacks involves inflammatory and immune responses, and, although most patients react positively to conventional treatments, in 20% of cases the symptoms persist [8]. In particular, the neutrophilic phenotype of asthma is the most commonly associated with corticosteroid resistance [6].

Considering these features, it is essential to identify new and alternative approaches to asthma therapy [3]. The development of strategies to reverse airway remodelling and to limit the progressive loss of lung function is important for patients resistant to standard therapies [9].

Regenerative medicine

An alternative approach to conventional drugs could be that of regenerative medicine, a novel strategy that uses mesenchymal stromal cells (MSCs) and

their derivatives (conditioned medium and extracellular vesicles) to stimulate repair mechanisms or regenerate tissues and organs.

Asthma-induced airway remodelling likely contributes to progression of asthma severity, leading in some patients to irreversible airway obstruction. Moreover, at inflammatory sites, the initial injury and the acute inflammatory response can result in apoptosis and necrosis of parenchymal cells, including endothelial and epithelial cells [10]. The repair process may involve replacement of these dead cells with MSCs that are able to differentiate in endothelial or epithelial cells. Alternatively, stem cell derivatives may drive paracrine mechanisms able to elicit the regenerative process.

There is abundant pre-clinical literature to support the use of cell therapy, including MSCs, in respiratory medicine, but there are few reports of clinical studies involving cell-based therapies for lung disease in humans [11]. An essential requirement to promote studies in regenerative medicine is the identification of an appropriate animal model for translational studies.

The horse: a unique animal model

To date, no animal model in which the response to regenerative therapy may be verified appropriately is

available. Before starting a clinical trial, the animal model has to match the clinical pathology in human. Indeed, the anatomy of the target organ and the possibility to translate the treatment response to humans are the main criteria to consider when selecting an adequate animal model [12]. Mouse models have been widely used to study the mechanisms underlying immune pathologies. However, most new therapies derived from mouse models have had limited success in clinical trials in humans [13]. In fact, the induction of asthma in mice fails to model the natural and chronic occurrence in humans, does not mimic the pathological changes associated with asthma and does not reflect the environmental factors that are present in the evolution of human asthma [14].

On the contrary, horses spontaneously develop “equine asthma”, a non-infectious chronic lower airway disorder sharing several similarities with human asthma [13,15]. In the search for the best possible experimental animal, the horse is considered a proper translational model for diseases such as tendon lesion because the equine superficial digital flexor tendon is similar to the human Achilles tendon [16,17], joint diseases like osteoarthritis [18] and respiratory pathologies precisely like asthma. Moreover, horses can be used as a model because of their size, athleticism, aging processes and spectrum of natural diseases. Also, multiple samples can be taken from the same animal without need for humane killing and a greater amount of sample material may be obtained, which could allow research to be conducted with fewer numbers of animals. In its severe form (severe equine asthma [SEA]), also known as “heaves” or “recurrent airway obstruction”, horses are affected by profound airways obstruction, leading to periods of respiratory distress at rest when susceptible animals are exposed to the offending antigens [13,19,20].

Horses and cats are the only animals that naturally develop asthma-type conditions such as seen in humans. However, unlike cats, the antigens responsible for the condition are known in horses, allowing the same animals to be studied under both exacerbation and remission states of the disease. In addition, SEA presents other important analogies with human asthma [21]. It is generally estimated that between 10% and 15% of adult horses suffer from this condition [22]. Both equine and human asthma are characterized by reversible bronchospasm, increased mucus production, airway hyper-responsiveness and remodelling of the airway walls [23]. In horses, these events are triggered, in genetically susceptible animals, by exposure to antigens, so an allergic response is considered pathogenetic of SEA, as seen in humans [24]. According to this hypothesis, a

Th-2–predominant immune response, as reported in human asthma, has been described in these horses [25,26]. Indeed, an increase in the Th-2 cytokine interleukin (IL)-4 and a decrease in the Th-1 type cytokine interferon- γ (IFN- γ) in bronchoalveolar lavage fluid (BALF) have been reported in SEA [27,28]. However, the immune response may be more complex in some asthmatic horses, in which IFN- γ , conversely, increased [29]. During clinical exacerbation, horses with SEA develop pulmonary neutrophilia [30], whereas increased mast cells and eosinophils are commonly observed in milder forms of equine asthma [20]. Similarly, the accumulation of eosinophils in the bronchi of human asthmatic patients is more common; neutrophilic inflammation is present as well, especially in those with severe and late-onset asthma [6]. Moreover, some authors showed an increase in Th-17 expression in horses with SEA that has also been demonstrated in people with neutrophilic asthma [31,32]. Human patients with neutrophilic asthma display an increased corticosteroid resistance [6] and serve as an important target for the development of new treatment approaches. Thus, the horse could represent a valid animal model for these patients.

As in human asthma, SEA results from multifactorial interactions between genetic predisposition and environmental factors. The interleukin-4 receptorA (IL4RA) has been reported as a candidate gene in SEA-affected animals [33]. Similarly, IL4RA polymorphism and alterations in the IL-4 receptor function are described in some phenotypes of asthma in humans [34]. In identifying an animal model to study human diseases, the homology of genes involved in immune function is crucial. In horses, some genes revealed greater homology with human compared with mice, such as *IL2* (72% horse versus 56% mouse), *IL23* (89% horse versus 77% mouse) and *IL17* (84% horse versus 75% mouse) [35]. Similarly, equine IFN γ -induced chemokine CXCL9, which is associated with Th1-type immune response, has 86% homology with humans, which in turn shows only 74% with mice [36]. In addition, the gene expression profile of equine macrophages after lipopolysaccharide (LPS) stimulation is more similar to humans than that of mice [37].

Leclerc *et al.* [23] showed other advantages of adopting the horse as an experimental model for human asthma. People and horses share a similar anatomy of the lung, and the equine model allows induction of bronchospasm and lower airway inflammation by exposure to antigens usually present in housing conditions for horses. Moreover, affected animals may also be studied during remission of the disease while at pasture or following administration of corticosteroids and bronchodilators.

Recognized advantages of the horse as animal model in respiratory diseases are also emphasized by Bullone and Lavoie [13]. First, SEA is a naturally occurring disease, rather than being induced by external manipulations such as in the rodent models. Furthermore, in severely affected horses, the condition is incurable, which is similar to chronic human asthma. The same authors [13] put the accent on other advantages of the equine model like its size and compliance of these animals. Bronchoscopy can be performed in standing sedated horses, allowing collection of an increased number of samples from all the respiratory tree, including tracheal wash (TW), BALF, endobronchial brushing and endobronchial biopsy. Studies on the role of granulocyte/macrophage colony-stimulating factor, which retards granulocyte apoptosis and contributes to the accumulation of inflammatory cells, have been made possible using BALF of horses [38,39]. Remodelling affecting the small distal airways can be studied over time and in the same individuals by means of large peripheral lung biopsy specimens collected using thoracoscopic surgery in standing sedated horses [40]. Finally, the chronic nature of the antigen exposure leading to asthma occurrence and

the lifespan of the horse (>25 years) are more similar to humans in comparison with other animal models such as the rodent, which rarely can undergo antigen hazard for more than a 3-month period [41,42]. Table 1 summarizes the similarity and the differences among human, mouse and horse in physiological and pathological conditions.

Asthmatic horses are also more likely to be better pre-clinical models for human asthma than the mouse model. A recent meta-analysis gave further evidence of the efficacy of inhaled corticosteroids, bronchodilators and chromones in the treatment of SEA, similar to that seen in human asthma [43]. Further similarities have been shown in the efficacies of novel therapies that promote a Th-1 immune response rather than a Th-2 response in both human and equine asthma [44,45]. The fact that some molecules, which showed promising results in the murine asthma models (Table 2), failed to be effective in humans demonstrates an increasing need for new models of asthma [13].

Despite its strengths as an asthma animal model, horses also have some weaknesses. Its large size requires higher costs of management, facilities and

Table 1. Similarity and differences in lung anatomy, etiology, pathophysiology and gene homology among human, mouse and horse.

	Human	Mouse	Horse (500 kg)
Lung anatomy			
Weight	1.3 kg (680 g right; 620 g left)	25 g	5 kg
Number of lobes	3 at right, 2 at left	5 at right, 1 at left	3 at right, 2 at left
TLC	6 L	1 mL	50 L
Blood-gas barrier thickness	0.62 μ m	0.32 μ m	0.6 μ m
Respiratory rate	10–14 bpm	250–350 bpm	8–16 bpm
Sub-mucosal glands	Present	Absence	Present
Distribution of bronchial artery	All the lung	Absent in pleura, septa and alveoli	All the lung
Airway smooth muscle	+++	+/-	+++
Etiology	Develops naturally	Antigen induced (e.g., ovalbumin)	Develops naturally
Pathophysiology			
Nonspecific airway hyper-responsiveness	Present	Present	Present
Inflammation	Persistent	Transient	Persistent
BAL cytology	Eosinophils Neutrophils Metachromatic cells	Eosinophils (Neutrophils)	Neutrophils Eosinophils (MEA) Metachromatic cells
Inflammation profile	Th2 and/or Th1/Th17	Depending on the antigen	Th2 and/or Th1/Th17
Clinical signs	Airway obstruction, cough, nasal discharge, wheeze Remission/exacerbation	Absent	Airway obstruction, cough, nasal discharge, wheeze Remission/exacerbation
Tissue remodelling	Persistent	Transient	Persistent
Duration of disease	All life	2–3 mo	All life (SEA)
Gene homology vs human			
IL-2		56%	72%
IL-17		75%	84%
IL-23		77%	89%
IFN γ -induced chemokine CXCL9		74%	86%

BAL, bronchoalveolar lavage; bpm, beats per minute; MEA, moderate equine asthma; TLC, total lung capacity.

Table 2. Efficacy of some treatments against asthma in human, mouse and horse.

Treatments	Human	Mouse	Horse
Corticosteroids	Yes	Yes	Yes
Anticholinergic agents	Possible	Yes	Yes
Anti-LTD4	Variable	Yes	No (SEA)
Antioxidants	Possible	Yes	Possible
Immunomodulatory	Low efficacy	Yes	Unknown efficacy
Antibiotics (macrolide)	Possible	Yes	Unknown efficacy
Cell therapy	No data	Yes	One article [41]

drugs, especially when compared with the mouse, limiting access to this model for academic research. But, in some cases, the horse could be the preferred model where translational research collaborations can be planned between universities that have animal science departments or schools of veterinary medicine and public/private medical or biological research centers where costs could not be prohibitive.

Moreover, few antibodies have been validated for this species, although the equine genome has been entirely sequenced, facilitating the identification of homologous sequences among different species to improve cross-reactivity [13]. Although horses are poorly suited for basic science research, naturally

occurring equine asthma could represent an important step to test therapies, potentially designed around mouse studies, before being translated to human trials.

Cell-based therapies

MSCs possess multi-differentiation abilities and immunomodulatory and immunosuppressive properties (Figure 2). To date, the use of MSCs in asthma therapy has been mainly investigated in the mouse. The most common allergen used was ovalbumin; the MSCs used were primarily bone marrow-derived (BM)-MSCs and MSCs and they were commonly administered intravenously (in a few studies intra-tracheally). In mice, MSC treatment for chronic asthma resulted in decreased airway inflammation, improved lung function, increased CD4+CD25+regulatory T-cell (T-reg) numbers and BALF as well as serum concentrations of IL-10 [46]. Histologically, the airways showed decreased thickening of the epithelial lining and reduced mucus hyper production and deposition of extracellular matrix. Moreover, MSC transplantation reduced airway remodelling and airway inflammation [5,9]. Srour and Thebaud [47], in their review of 19

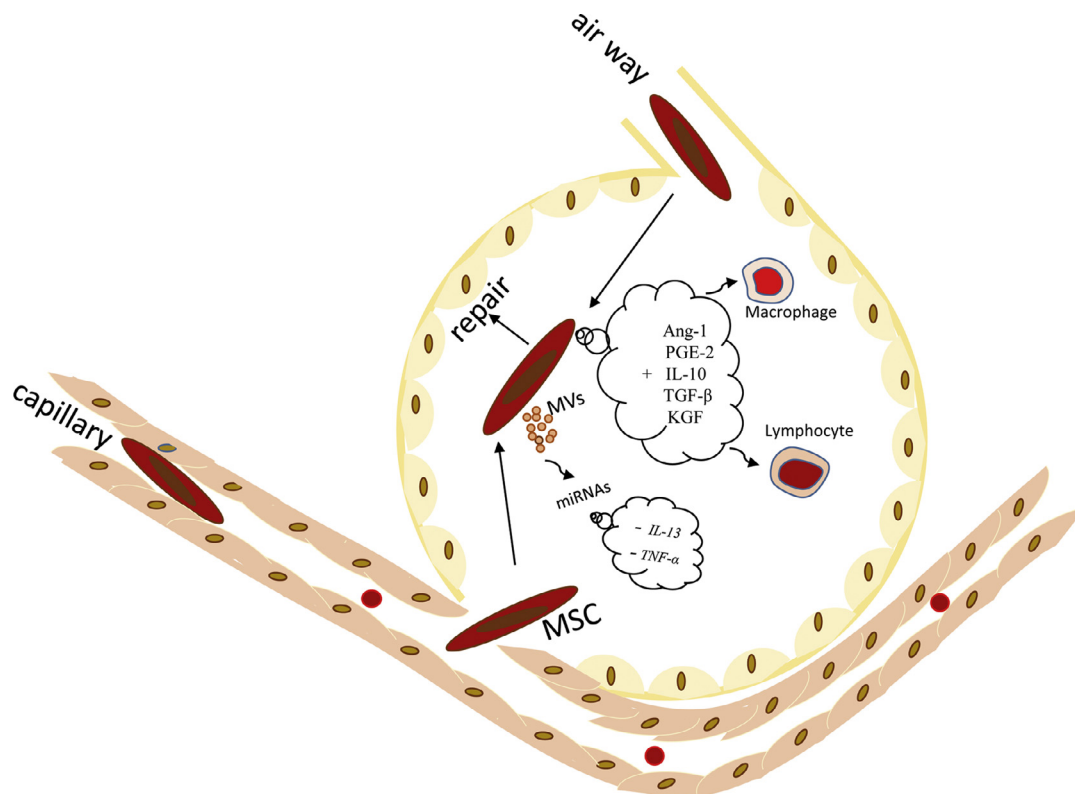


Figure 2. Hypothetical mechanism of action of MSCs inside the alveolus. MSCs can be administered intravenously or intra-tracheally and can act by repair or by paracrine mechanism. They secrete soluble factors (angiotensin-1 [Ang-1], prostaglandin E-2 [PGE-2], IL-10, transforming growth factor [TGF]-β, keratinocyte growth factor [KGF]) or in-soluble factors such as extracellular vesicles, which, by miRNAs, are able to down-regulate the expression of some pro-inflammatory genes such as *IL-13* and tumor necrosis factor (*TNF*)-α.

reports investigating the effect of MSCs in an asthmatic mouse model, reported that, in most studies, IL-4, IL-5, IL-6, IL-9 and IL-13 levels in BALF were decreased with MSC treatment. The effect of MSC treatment on BALF IFN- γ was variable with a significant decrease observed in four studies.

These beneficial effects may be mediated by up-regulation of transforming growth factor- β (TGF- β) through activation of the signal transducer and activator of transcription 6 (STAT6) pathway [48]. This action of MSCs appeared to derive from the promotion of the Th1 phenotype, the inhibition of the Th2 phenotype and the up-regulation of T-reg [48]. A study by Ge *et al.* [49] showed up-regulation of Th1-derived cytokines and suppression of Th2-derived cytokines when MSCs were administered in asthmatic mice. To date, only one study has tested the efficacy of BM-derived mononuclear cells in horses with SEA [50]. Cells were administered intra-tracheally, which in horses can be easily accessed using endoscopy. Moreover, the intra-tracheal route allows delivering a higher number of cells directly into the airways [51]. The results of this study were consistent with those obtained in mice: a reduction of clinical signs and an increase of the levels of the anti-inflammatory cytokine IL-10. Moreover, as reported by previous observations in horses treated with conventional therapy, the number of neutrophils decreased and the number of macrophages increased in BALF. The difference between conventional therapy and cell therapy is that the former only promotes regression of the inflammatory process but cannot reverse the chronic airway remodelling, whereas cell therapy may protect or even repair the damaged tissue and reduce the response to allergens [50].

MSCs and immunomodulatory effect

The beneficial effects of MSCs result from repair and regeneration. Goldstein *et al.* [52], in a study performed *in vivo* in the mouse and *in vitro* on murine airway fibroblast cells, demonstrated that human MSCs influence remodelling by decreasing extracellular matrix deposition as well as collagen I, collagen III and hyaluronan synthesis. However, to date, the main effect of MSCs is attributable to the reduction of the inflammatory response. It is known that MSCs can act on lung inflammation by modulating the immune system [11]. Indeed, in studies with bleomycin in a murine model, MSC administration immediately after exposure significantly reduced inflammation and collagen deposition associated with the lung fibrosis, despite the low rate of engraftment [5,53,54]. Interestingly, other recent data suggested that, despite the scarce cell engraftment in the lung, treatment with systemic or intrapulmonary administration of BM-derived mature

MSCs in mice decreased mortality, ameliorated alveolar fluid clearance and reduced inflammation and lung injury [55]. These results suggest that the mechanism of MSC action in the murine model of pulmonary fibrosis was not due to cell engraftment into the injured tissue but it was hypothetically associated with the secretion of soluble or insoluble factors from MSCs that stimulate healing [5]. This hypothesis is strengthened by many data suggesting that the conditioned medium (CM) secreted by MSCs during culture may have an overlapping effect with that of the MSCs administered in different lung injury models [56,57 (review)]. It is possible that MSCs could exert their effects through a paracrine effect rather than by lung engraftment [46,57,58]. MSCs have the ability to modulate the immune system by the release of pro-inflammatory and anti-inflammatory cytokines and lipid mediators, such as prostaglandin E-2 (PGE-2). In addition, MSCs also secrete angiopoietin-1 (Ang-1), with an effect on endothelial barrier, and growth factors, which have cytoprotective and repair abilities, including keratinocyte growth factor (KGF) [57]. In both acute and chronic models, CM prevented airway hyper-responsiveness and inflammation, airway smooth-muscle thickening and peribronchial inflammation [59]. Significant reductions in the Th2 cytokines IL-4 and IL-13 and a significant increase in IL-10 were found along with an increase in IL-10 excreting T-reg lymphocytes [59]. Arudda de Faria *et al.* [60] proposed that the action of MSCs could take place through the expression of antagonists of IL-1 receptors or of tumor necrosis factor (TNF)- α and other pro-inflammatory factors that activate resident macrophages. In addition, an increase of the expression of protective factors, such as antiproteases, is hypothesized. Thus, it seems that effects of MSCs are mostly (but not entirely) mediated through soluble factors rather than by MSCs acting as precursors to mesenchymal structural cells [47].

MSCs and extracellular vesicles

In addition to the paracrine activities of soluble factors present in CM, different hypotheses suggest that also non-soluble factors, like extracellular vesicles (EVs), allow MSCs to communicate with target cells, influencing their behavior [11,61].

Two distinct populations of EVs have been identified: those originating from multivesicular bodies (exosomes) between 30 and 120 nm in diameter, and vesicles that bud from cell membranes with a variable size (80 nm–1 micron; shedding vesicles) [62]. The cell-surface markers of EV include receptors that allow them to bind to cell surfaces. After binding, the EV membrane coalesces target cell and the contents of the EVs are released into the cell

cytoplasm [63–65]. It has been shown that EVs promote tissue repair, both *in vitro* and *in vivo* [66–68], probably because they are involved in many physiological functions, such as immunity [69], signaling [70], angiogenesis, extracellular matrix, remodelling [71] and even gene regulation [72]. The effect of EVs in these physiological functions suggests their use in cell-free therapies. Indeed, in addition to the administration of MSCs, these findings support the use of only CM or purified EVs for regenerative treatment of damaged tissues [73]. In some pre-clinical studies of lung diseases, EVs derived by MSCs were effective in improving pulmonary hypertension and acute lung injury in murine and mouse models [74–77]. Often, the issue with these animal models is the method used to induce the allergic airway inflammation, such as, for example, by repeated mucosal exposure to *Aspergillus* hyphal extract. Regarding the horse as animal model, only one article investigated *in vitro* the effects of CM and EVs derived from amniotic progenitor cells on equine alveolar macrophages (AMs) stressed by LPS [73]. The AMs were collected by BALF and stimulated with LPS that induces lung inflammation either *in vivo* or *in vitro* [55,78–80]. Both CM and EVs significantly reduced the release of TNF- α , TGF- β and IL-6 induced by LPS. In addition, these results showed that CM acted more rapidly than EVs. The soluble factors of CM can act immediately but it is conceivable that EVs require internalization and act later through modification of gene transcription and translation [70,81]. Indeed, EVs contain a different kind of RNA species, including microRNAs (miRNAs) [82] that are able to down-regulate *IL-13* expression or to inhibit the production of some pro-inflammatory cytokines in an animal model with induced asthma [83,84]. These data support the potential use of CM and EVs in the treatment of horse pathologies, including airway diseases in which TGF- β may be detrimental, such as respiratory allergy [73]. TGF- β is primarily known as an anti-inflammatory cytokine, but Zucca *et al.* [73] showed that TGF- β alone could induce TNF- α release. Other researchers report the ability of TGF- β to induce disease, for example, in immune-related cells [85–88]. The ability of CM and EVs to oppose the secretion of pro-inflammatory cytokine induced by LPS, including TGF- β , may be useful in all the situations in which it has a role [73].

Soluble and insoluble factors have also been studied in reduction of bleomycin-induced lung fibrosis in mice [89], supporting the potential use of secretome in lung-regenerative medicine. Future developments could include the administration of EVs, by aerosolization or intranasally, or the direct transfer of miRNAs with anti-inflammatory effect. These

solutions could overcome the problems of the use of cells, including the clumping of MSCs in the pulmonary vasculature when administered intravenously, and the immunogenic response following heterologous treatment.

To our knowledge, no trial has yet examined EVs-MSC transfer in spontaneous asthma and this article suggests the treatment of inflammation and remodelling in severe equine asthma, by cells, CM or EVs, as a model for the human disorder.

Conclusions

Many challenges remain in the prospective use of cell therapy in spontaneous asthma. Better understanding of the potential actions of MSCs is required and, yet, there are no established protocols for MSC infusion in horses. Previous murine models have administered between 0.1 and 2.0×10^6 cells per infusion (average $\sim 0.5 \times 10^6$). Future studies must establish the quantity of cells or CM or EVs required when evaluating their therapeutic effects in equine lung diseases, taking into account the lung mass of horses when compared with mice.

In conclusion, most of the features of human asthma (clinical presentation, pathological changes and immunologic response) are also present in equine asthma. For this reason, we suggest that the horse could represent an ideal animal model for human asthma and could facilitate more rapid development of cell-based therapies for this debilitating disease.

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