Inhaled conditioned cell culture media: animal model for treatment of equine asthma

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Inhaled conditioned cell culture media: animal model for treatment of equine asthma

Abstract

The study focuses on exploring novel treatments for severe equine asthma (SEA), a chronic inflammatory condition that shares similarities with human asthma. SEA is associated with environmental, genetic, and immunological factors, offering a valuable model for investigating airway inflammatory processes. In this context, the use of inhaled conditioned media derived from mesenchymal stem cells (MSC) was explored as a potential treatment for SEA. MSC were stimulated with Toll-like receptor ligands and immune-related factors, resulting in two types of supernatants: polarized and non-polarized. First, a safety assessment was conducted in mice, showing no significant differences in respiratory mechanics between treated and control groups. Subsequently, a clinical trial was conducted in horses diagnosed with SEA, evaluating clinical and cellular responses following treatment with MSC supernatants. The clinical responses were positive, with stable cell counts and a notable reduction in neutrophils in some horses after 10 days of treatment with polarized conditioned medium. Despite these promising results, the study concludes that further trials are required to validate the specific efficacy of this treatment in horses. Moreover, the equine model provides valuable insights that could bridge the gap between rodent studies and human trials, suggesting that this approach might be beneficial in managing human asthma.

Keywords: Asthma; Mesenchymal stem cells; Supernatant; Conditioned medium; Equine model.

Study contribution

This preliminary study aims to provide a methodological framework for the treatment of equine asthma using stem cells. The methodology was designed to evaluate the safety of the treatment and its potential therapeutic effects. For this purpose, both bronchoalveolar lavage cellular parameters and clinical signs were assessed in the animals studied.

Introduction

Asthma is a chronic inflammatory disorder of the airways, prevalent worldwide, leading to diminished quality of life, disability, and even mortality. This condition, marked by symptoms such as wheezing, shortness of breath, and coughing, arises from complex immunological and inflammatory reactions. Current mainstream treatment in humans involves corticosteroid administration, which, while reducing airway obstruction and enhancing quality of life, often fails to fully alleviate symptoms or reverse the underlying pulmonary pathology asthma. The persistent nature of these symptoms, despite treatment, underscores the need for innovative therapeutic approaches.[1]

In the quest to identify new targets for human asthma therapy, animal models have been indispensable. Traditionally, rats and mice have been used in experimental settings to induce disease-like conditions. However, the exploration of alternative animal models is gaining momentum, particularly the use of horses suffering from naturally occurring asthma. This condition in horses, especially severe equine asthma (SEA), presents significant parallels with human asthma, characterized by reversible bronchospasms, neutrophil accumulation in the lungs primarily mediated by Th2 responses, and similar patterns of pulmonary remodeling. This

remodeling in SEA closely mirrors that in human asthma, positioning the equine model as a unique avenue for investigating tissue repair and evaluating new therapeutic interventions. Furthermore, the equine model offers a distinct opportunity to test therapies initially designed using rodent models before transitioning to human trials. The remarkable similarity in inflammatory responses and airway remodeling patterns between human and equine asthma suggests that insights gained from treating equine asthma could directly inform the management of the human condition.[2]

Inflammatory processes of the lower respiratory tract represent a significant pathology in equine medicine.[3] These conditions are typically multifactorial, encompassing allergic and infectious etiologies, as well as exposure to various irritants or toxic elements. Environmental pollution, including gases such as ammonia and methane from organic waste, together with allergens like dust mites and β-D-glucans—components of fungal and bacterial cell walls—play a crucial role in eliciting pulmonary allergic immune responses in horses.[4]

The intricate relationship between inflammation and hypersensitivity in these pathologies stems from the exaggerated expression of genes encoding inflammatory mediators. This gene expression is contingent upon the activation of various transcription factors, with the NF-κB signaling pathway being instrumental in inducing the transcription of several genes involved in this disease process, including those coding for chemokines, cytokines, cell adhesion molecules, and leukocytes.[5]

Clinically, these diseases are most prevalent in middle-aged or elderly equines, characterized by symptoms such as coughing and respiratory dyspnea, often worsening

seasonally and upon exposure to specific triggers. Advanced cases may progress to weight loss, pulmonary emphysema, and hypertrophy of the respiratory muscles. Diagnosis primarily hinges on clinical signs such as respiratory hyperreactivity, abnormal lung auscultation, and an increased percussion field, complemented by tracheal-bronchial endoscopy, bronchoalveolar lavage (BAL), percutaneous transtracheal aspirate (TA), and cytological analysis of respiratory secretions. Other more sophisticated assessment methods include intrathoracic pressure measurement and arterial blood gas analysis. Inflammatory cells—predominantly alveolar macrophages—dominate the cell counts in TA and BAL, particularly in stabled animals.[6, 7] Generally, TA should contain less than 20 % neutrophils, 10 % lymphocytes, and a minimal presence of mast cells and eosinophils. BAL typically exhibits 30-60 % lymphocytes and 40-70 % macrophages, with neutrophils, mast cells, and eosinophils comprising 5 %, 2 %, and 1 % respectively.[8] Neutrophil characteristics, such as degeneration and toxic changes, suggest infection, while apoptosis indicates inflammatory airway diseases like asthma. The presence of hemosiderophages often reflects hemorrhage in the lower respiratory tract.[9]

The cornerstone of pharmacological treatment for these conditions involves anti-inflammatories, bronchodilators, and mucolytics. Traditionally administered orally or parenterally, there is a growing trend towards inhalation therapy to enhance therapeutic efficacy and mitigate side effects.[10] This approach necessitates an effective nebulization system to ensure that the therapeutic agent reach the lower airways. Metered-dose inhalers are increasingly becoming fundamental in these treatments. Commonly used anti-inflammatories include glucocorticoids such as dexamethasone and prednisolone, which are effective both orally and parenterally. The inhaled glucocorticoids of choice are often beclomethasone and

fluticasone. Clenbuterol, a bronchodilator, is preferred due to its superior efficacy and fewer side effects and is available for oral or parenteral administration. Other inhalable bronchodilators include salbutamol and ipratropium bromide.[11]

Cell therapy, particularly focusing on specific adult stem cell types, is emerging as a promising therapeutic strategy for the treatment of asthma in horses. Mesenchymal stem cells (MSC) are favored due to their significant immunomodulatory capacity and relative ease of *ex vivo* manipulation and expansion. These cells can secrete a variety of soluble factors such as hormones, cytokines, chemokines, and growth factors.[12, 13] Recent studies have validated the effectiveness of this therapy, not only in improving bronchoalveolar lavage fluid (BALF) cytological profiles but also in increasing IL-10 levels, which inhibit the production of proinflammatory cytokines such as TNF.[14] This treatment significantly contributes to reducing airway remodeling, offering protection and potentially promoting the repair of affected tissues, thereby improving allergen tolerance.

A novel aspect of our hypothesis involves enhancing MSC *in vitro* to promote the production of increased quantities of anti-inflammatory molecules and exosomes. Prioritizing MSC polarized with Toll-like receptor (TLR) ligands and immune factors is crucial, as this type of activation enhances the MSCs' immunosuppressive phenotype.[15]

Based on this background, the present study aimed to evaluate the safety of using inhaled supernatants derived from polarized equine MSCs as a treatment for SEA. A preliminary safety trial was conducted using a murine model.

This therapy aims to modulate the cellular response during the inflammatory process, influencing the cellular profile and cytokine secretion, to achieve a potential anti-inflammatory effect.

Materials and methods

Ethical statement

The study was approved by the Comité de Bioética of the Universidad Alfonso X el Sabio (UAX) on December 9, 2021, and registration number 2021-12/109.

Isolation and expansion of equine Mesenchymal Stem Cells

The donor animals used for obtaining MSCs for this study were patient horses at the UAX Veterinary Clinical Hospital. Their ages ranged between 4 and 15 years, with variation in sex and breed. The samples were obtained in accordance with animal welfare regulations and with the informed consent of the owners. The adipose tissue samples were collected during colic surgery, consisting of intra-abdominal adipose tissue weighing between 10 and 30 g, and transported under refrigeration at 4−8 °C in a sterile container. All samples were processed within 24 hours.

The isolation of MSCs was carried out from abdominal adipose tissues. After multiple washes with PBS, the tissue was minced into small fragments and subjected to enzymatic digestion at 37 °C for 45 minutes with 0.1 % type II collagenase. Following digestion, the mixture was filtered (70 µm nylon filters; FALCON), diluted in a 1:4 ratio with PBS, and centrifuged at 1 200 rpm for 5 minutes, thereby obtaining the stromal vascular fraction (SVF). The SVF was seeded at a density of 10^8 cells/cm^2 in Dulbecco's modified Eagle's medium

(DMEM, Corning®) supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine, 1 % penicillin, and 1 % streptomycin. The cells were incubated at 37 °C in a 5 % CO_2, humidity-saturated atmosphere. When the cultures reached 80−90 % confluence, the cells were treated with 0.25 % trypsin-EDTA for 5 minutes at 37 °C. The detached cells were then reseeded on a larger culture surface. From the third passage onward, a homogeneous population of MSC was typically obtained, characterized by their defined fibroblastic morphology, specific surface marker expression (negative for CD34, CD11b, and CD45; positive for CD90, CD44, and CD29), and a marked differentiation towards mesenchymal lineage using specific induction media.

Immunophenotyping of isolated MSC

The determination of the MSC immunophenotype was performed using flow cytometry with the following monoclonal antibodies conjugated to fluorochromes: CD29 (Biolegend), CD34 (Becton Dickinson), CD44 (ABCSerotec), CD79a (AntibodyBCN), MHC-II (ABCSerotec), CD45 (Biolegend), CD90 (AntibodyBCN), and CD105 (ABCSerotec). Horse MSC (3×10^5 cells) were resuspended in PBS and incubated with the monoclonal antibodies at 4 °C for 30 minutes. After washing, the labeled cells were resuspended in PBS and analyzed in the MACSQuant flow cytometer (Miltenyi Biotec). Data acquisition was performed with the MACSQuantify software (Miltenyi Biotec).

In vitro enhancement and polarization of Mesenchymal stem cells

MSC polarization was carried out at the fourth cellular culture passage. The MSC were seeded under standard conditions and, once the required confluence was reached, the selected

polarizing agent was added: Poly:IC (10 µg/mL; polyinosinic–polycytidylic acid sodium salt, Merck) or IFN-γ (10 ng/mL; recombinant equine IFN-gamma protein; R&D Systems, Minneapolis, MN, USA) combined with TNF-α (10 ng/mL; recombinant equine TNF-alpha Protein, R&D Systems, Minneapolis, MN, USA), and incubated for 1 or 24 h, respectively, at 37 °C in a 5 % CO_2 and humidity-saturated atmosphere. After stimulation, two PBS washes were performed to remove FBS residues, and phenol red-free DMEM culture medium was added. At 24 h post-production, the supernatant was collected for treatment recovery, and the cells were harvested for gene expression analysis. MSC maintained in DMEM culture without polarizing agents were used as a negative control. The supernatant was concentrated using centricons (Pierce Protein Concentrator PES; Thermo Scientific) with a 10 kDa molecular weight cut-off of and centrifuged at 4 700 × g for 25 minutes at room temperature to a final volume of 15 mL. The resulting treatment was stored at -80 °C until its administration in equines.

Analysis of gene expression in MSC

In order to determine the immunomodulatory phenotype of MSC, gene expression analysis was carried out using cells polarized with Poly I:C or IFN-γ in combination with TNF-α. Once the cells were collected, total RNA (tRNA) extraction was performed using the High Pure RNA Tissue kit (Roche), following the manufacturer's instructions. The cells were lysed and incubated with the retention membrane, treated with deoxyribonuclease I (DNase I; Roche), and, after several washes, finally eluted. The concentration and integrity of the tRNA were determined using a UV-visible spectrophotometer (UV/VIS) for nucleic acid quantification (NanoDrop One Spectrophotometer: Thermo Scientific). The extracted tRNA was used as a

template for the synthesis of complementary DNA (cDNA). cDNA synthesis was carried out using the PrimeScript™ RT Reagent Kit (Takara) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was then performed using the synthesized cDNA, with SYBR Green as the fluorochrome (TB Green™ Premix Ex Taq; Takara) and gene-specific primers. The genes amplified by this PCR are listed in **Table 1**, along with the primers used for amplification (Sigma-Aldrich).

Table 1. PCR-Amplified Genes and Primers Used (Sigma-Aldrich)

GENE	NAME	NCBI Reference Sequence	Sequence primer (5´ 3´)	Tm
IDO	Indoleamine 2,3-dioxygenase 2	XM_014736539.2	F: TCATCTCCACCGGCTATGTC R: GTTTGTCAGCACCAGGTCTG	59
NOS2	Nitric oxide synthase 2	NM_001081769.1	F: AATCCTCTTTGCCACGGAGA R: TCGTCCATGCAGAGAACCTT	59
iNOS	Inducible nitric oxide synthase	NM_001081769.1	F: CCAACAATGGCAACATCAGGT R: TGAGCATTCCAGATCCGGA	59
IL-6	Interleukin-6	EU438770	F: AACAGCAAGGAGGTACTGGCA R: CAGGTCTCCTGATTGAACCCA	59
PTGS	Prostaglandin-endoperoxide synthase 2	NM_001081775.2	F: AAAGGCCTCAATCGACCAGA R: CGCAGCCATTTCCTTCTCTC	59
COX-1	Prostaglandin-endoperoxide synthase 1	DQ246452	F: TCTATGCTACGCTCTGGCTACG R: TTGATGGTCTCCCCGATGA	59
COX-2	Cyclooxygenase-2	AB041771	F: GTTTGCATTTTTTGCCCAGC R: ACTTAAATCCACCCCGTGACC	59
IL-10	Interleukin-10	JQ432545.1	F: AAAAGCTGAAGACCCTCCGA R: ACTCTTCACCTGCTCCACTG	59
SCAMP3	Secretory carrier-associated membrane protein 3	XM_001498730.6	F: GGTTGCCCTGTTCTTCACTG R: GGAAAAGACACCAGCAGCAA	59
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001163856.1	F: TGCCCCAATGTTTGTGATGG R: CACTGTGGTCATGAGTCCCT	59

The amplification program consisted of an initial denaturation at 95 °C for 1.5 min, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at 60 °C for 50 seconds. Melting curve analysis included one cycle at 60 °C for 1 minute, followed by a temperature

increase of 0.1 °C/s until reaching 95 °C. qPCR was performed on the Quantstudio 3 thermocycler, and relative gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method, with SCAMP3 and GAPDH as internal normalization (housekeeping) genes.

Safety profiling and pulmonary function testing in mice

To determine the safety profile of repeated inhalation of conditioned medium and its potential impact on lung function, a C57BL/6 mouse model was employed. Five mice per group in the control and treated groups received intranasal instillations of conditioned medium or phosphate-buffered saline (PBS), using an instillation volume of 12.5 µL/nare, once per week for a total of 3 weeks. Animals were evaluated after an additional week of washout.

FlexiVent equipment (SCIREQ) was employed for rodent mechanical ventilation and pulmonary function testing using the forced oscillation technique. Data were collected using a linear single-compartment model and a broadband frequency constant-phase model to stablish lung function parameters. The animals were anesthetized with 4 % inhaled isoflurane (Forane®; Abbott Laboratories) and subjected to tracheotomy with standardized tracheal cannula (EMMS). Neuromuscular blockade was induced with 0.5 mg/kg of intraperitoneal atracurium besylate. Mechanical ventilation was set at 250 breaths per minute with a tidal volume of 0.14 mL and 2 cm H_2O positive end-expiratory pressure Lung mechanics parameters were measured.

Statistical analysis

Statistical analysis was performed using Prism 8 software (GraphPad, La Jolla, CA). Statistical significance, defined as $P < 0.05$, was determined using unpaired t-tests for variables with normal distribution.

Mice bronchoalveolar lavage and mice lung tissue processing

Following pulmonary function recordings, animals were euthanized under deep anesthesia by intravenous injection of 0.3 mL of 7.45 % potassium chloride (Braun). BAL was immediately collected through the tracheal tube using 2 mL of saline in 1mL fractions. BALF was centrifuged at 500 × g for 10 min. The resulting cell pellets were analyzed, and total live leukocytes were counted using a Neubauer hemacytometer with trypan blue dye exclusion.

Immediately after BALF collection, the pulmonary vascular circuit was flushed with 2 mL ethylenediaminetetraacetic acid (EDTA) in PBS, administered through the right ventricle and drained via an incision in the abdominal aorta. The cardiopulmonary block was then dissected, inflated, and fixed with 10 % formalin. From the inflated and fixed lungs, sagittal slices were obtained, placed into standard tissue processor cassettes, and paraffin embedded with the lung's mid-sagittal plane facing the microtome blade. Paraffin blocks were sectioned into 5-µm-thick slices and stained with hematoxylin and eosin (H&E) for general histopathological evaluation.

Horses clinical study

A total of 12 Spanish Sport Horse jumpers (males, n = 12; females, n = 0), aged between 2 and 24 years, were selected for the study. They were housed in equestrian centers in the province of Madrid and at the Veterinary Medicine Faculty of UAX. Of these, 3 horses served as an asymptomatic control group, and 8 showed clinical signs of asthma. Horses with symptoms were treated and sampled at days 3 and 10 post-treatment. The animals were housed under similar conditions: stabled in individual, well-ventilated boxes, with wood shaving bedding. Their diet consisted of three daily rations of feed and grass hay, which was provided on the ground, and they had ad libitum access to water. A clinical history was recorded for each horse, including data on age, sex, breed, and activity. Anamnesis including housing type, feed type and administration method, recent transport history, and previous clinical history —particularly any respiratory conditions or low performance. All animals underwent a complete physical examination, wich included general condition, demeanor, rectal temperature, lymph nodes palpation, and cardiac and abdominal auscultation. Special attention was paid to the respiratory system, evaluating breathing frequency, pattern, rhythm, and depth; cough reflex; nasal discharge; and the presence of a respiratory effort line. Blood samples were collected for hematology and biochemistry. Finally, a bronchoalveolar lavage (BAL) was performed for macroscopic evaluation and differential cell count. The criteria used to define mild-moderate asthma were: > 10 % neutrophils, > 5 % mast cells and > 5 % eosinophils. Severe asthma was defined as > 25 % neutrophils in BAL.

Treatment consisted of administering 15 mL of concentrated MSC-derived supernatant (obtained from 200 mL of conditioned medium from a 100 million MSC culture) to each horse. Delivery was performed via a Flexineb 2 nebulizer, chosen for its wireless, nearly silent design,

which facilitated handling without requiring sedation. The supernatant was aerosolized, enabling efficient delivery into the lower respiratory tract. Each 15 mL dose required approximately 15 minutes to nebulize.

For the BAL procedure, the horses were sedated using a combination of detomidine and butorphanol. Once sedated, a 3-meter-long Cook® catheter was used. The catheter was inserted through one nostrils—previously cleaned with neutral soap—an advanced through the larynx until it reached the carina, stopping where the bronchial diameter no longer allowed further progression. At this location, 300 mL of pre-warmed physiological saline was instilled. The fluid was infused and aspirated using 60 mL syringes, recovering more than 50 % of the instilled volume. To reduce coughing, which is common during the procedure, 1 % lidocaine (5 mL) was administered upon reaching the larynx, and another 5 mL at the carina. The recovered fluid was was stored under refrigeration until processing within a few hours of collection. Samples were centrifuged at 1 500 rpm for 10 minutes, and the cell pellet was smeared and stained using the rapid Diff-Quick® staining technique. For the differential cell count, 15 fields per slide were examined under the microscope, and the percentage of each nucleated cell type was recorded.

Each horse underwent an initial BAL just before therapy administration. Depending on the assigned group, a second BAL was performed either 3 or 10 days later to assess cellular response to treatment. Horses were monitored for adverse reactions in the hours immediately following administration, and daily physical examinations were performed thereafter.

Results

Characterization of equine mesenchymal stem cells derived from adipose tissue

Cells harvested from equine abdominal adipose tissue cultures at 80−90 % confluence in the fourth passage were used for flow cytometric analysis of surface antigen expression. The isolated cell population co-expressed mesenchymal stem cell (MSC) markers: CD29, CD44, CD79a, CD90, and CD105. They were negative for hematopoietic markers CD34, CD45, and MHC-II (**Figure 1**).

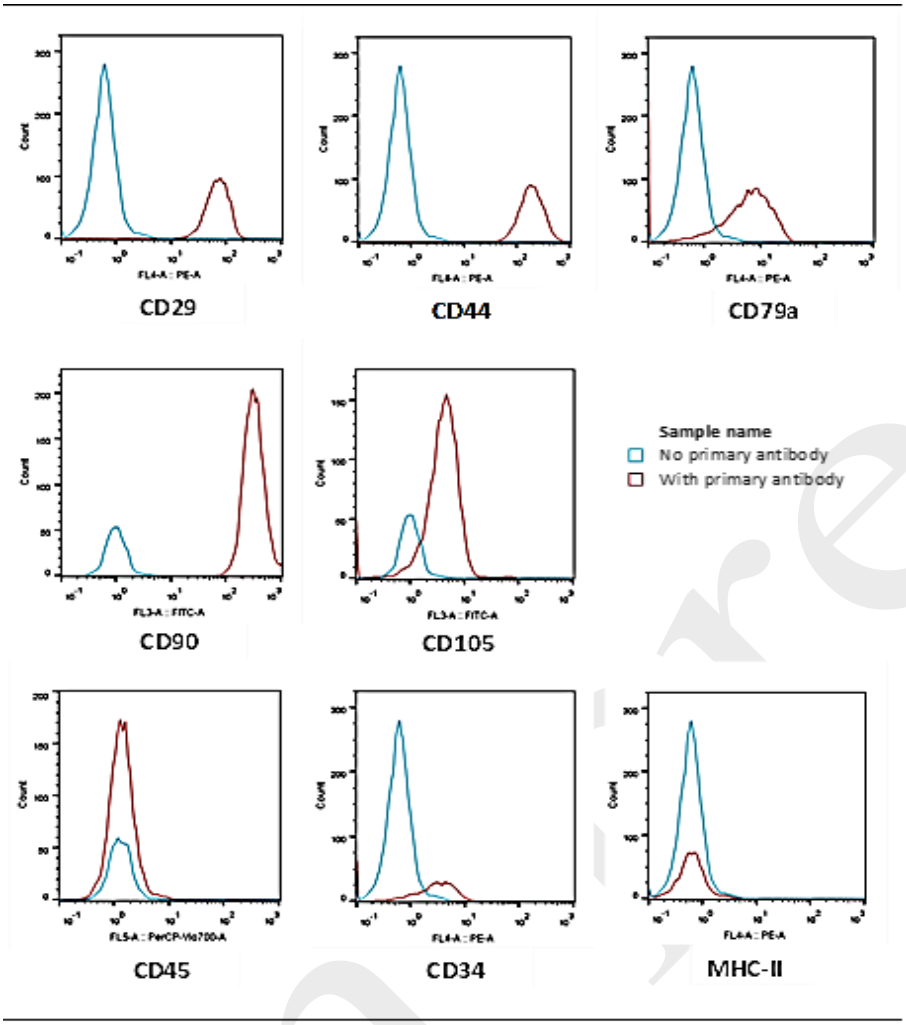


Figure 1. Phenotype characterization of the isolated equine mesenchymal stem cell (MSC) population. Flow cytometry histograms representing the immunophenotyping of the MSC: positive for the surface cell markers CD29, CD44, CD79a, CD90, and CD105, and negative for CD45, CD34, and MHC-II.

Effect of priming Mesenchymal Stem Cells on gene expression

To assess the impact of priming mesenchymal stem cells with polarizing agents on the expression of genes associated with anti-inflammatory activity, we analyzed the relative expression of the following genes: IDO, iNOS, NOS2, PTGS, IL-6, IL-10, COX-1, and COX-2. MSC stimulated with IFN-γ and TNF-α showed an increased expression of IDO, NOS2, iNOS, IL-6, and COX-2, although statistical significance was observed only for IL-6 and iNOS (**Figure 2**). In contrast, MSC stimulated with Poly:IC showed elevated expression levels of PTGS and COX-1, but these changes did not reach statistical significance. No significant differences were observed in IL-10 expression following stimulation with either of the polarizing agents (**Figure 2**).

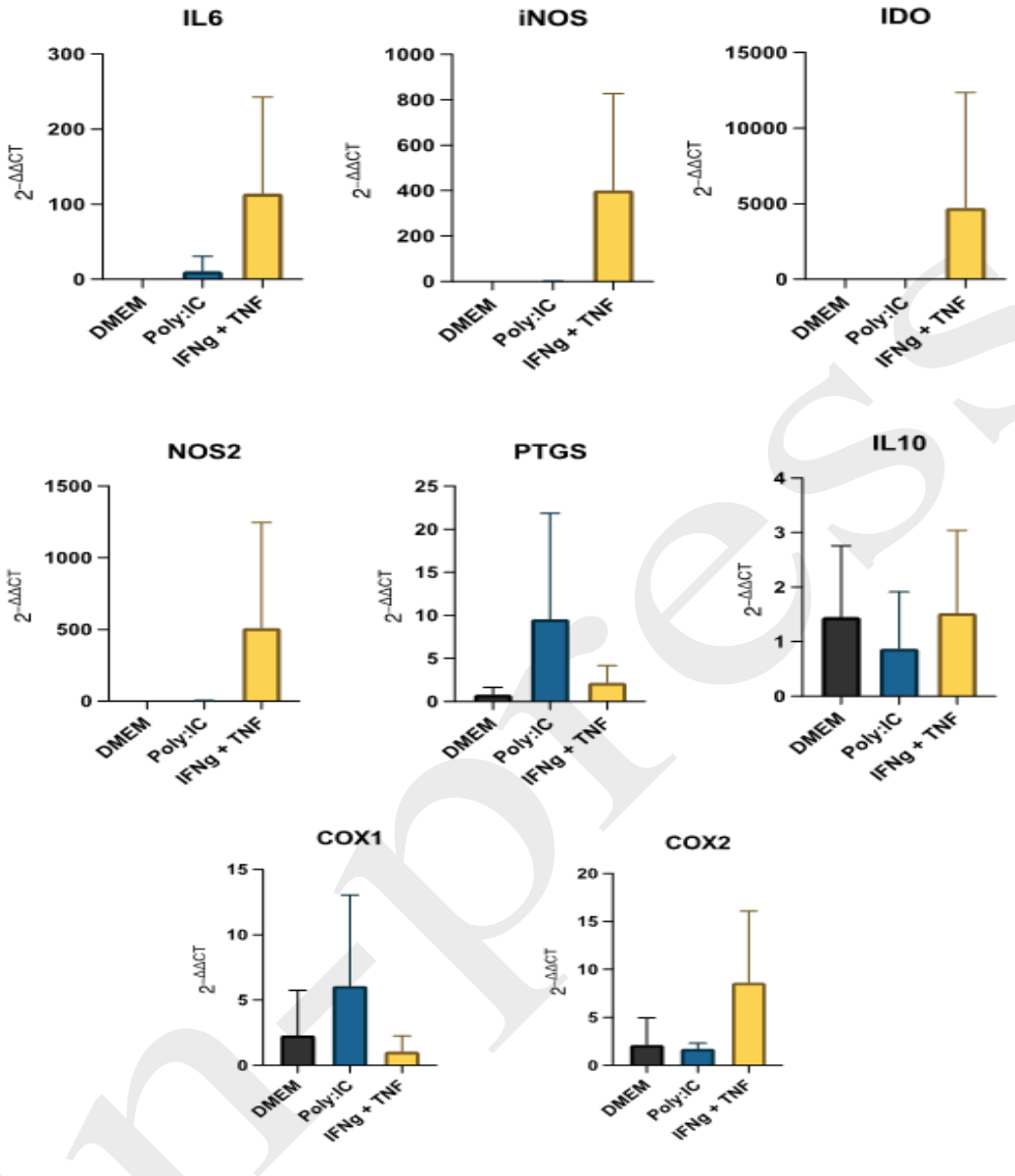
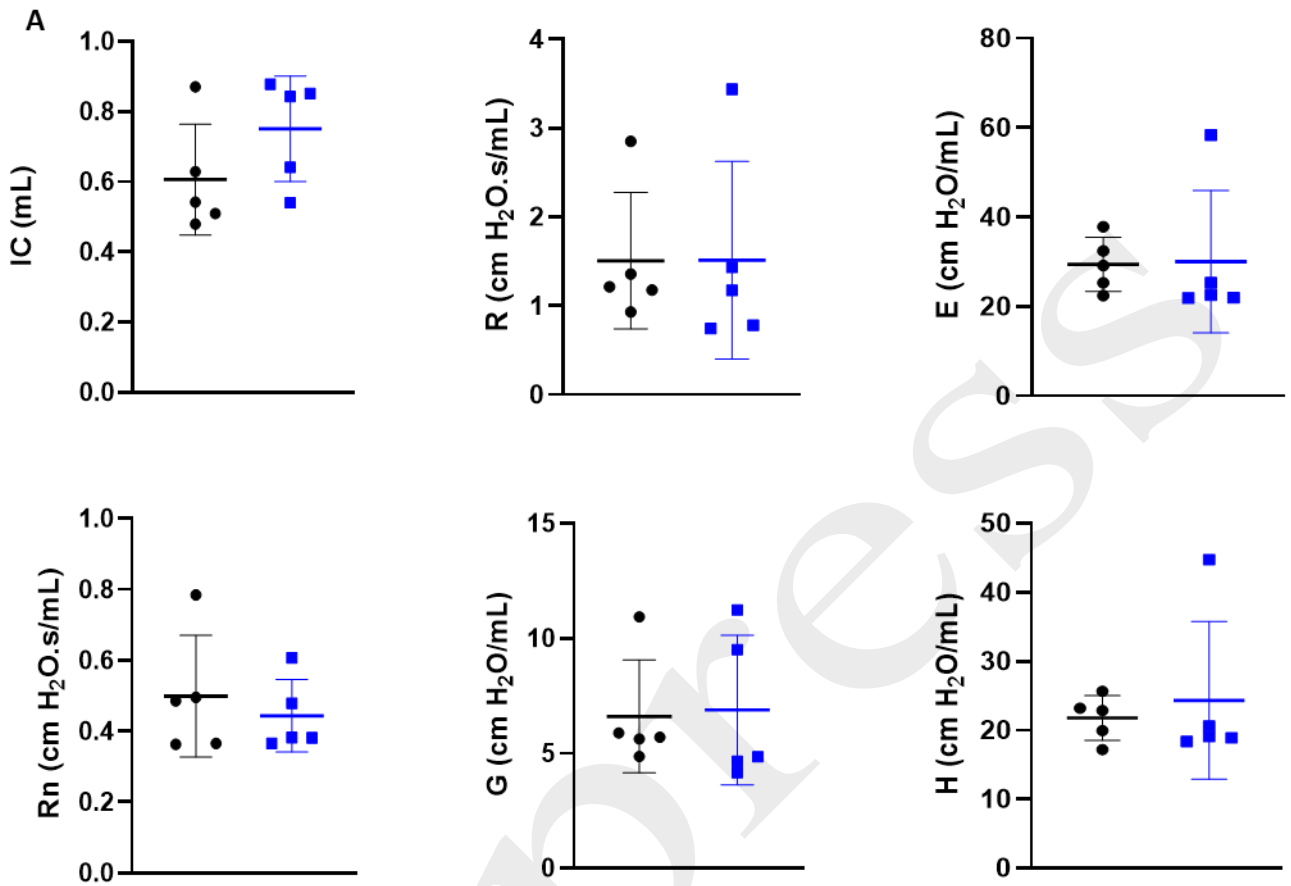


Figure 2. Analysis of maker expression in mesenchymal stem cell (MSC) induced by immunomodulatory molecules. MSC were incubated with Poly:IC (10 µg/mL) or IFN-Ɣ (10 ng/mL) combined with TNF-α (10 ng/mL). 24h following incubation, the mRNA levels were analyzed by RTqPCR.

Interleukin-6 (IL-6). Inducible nitric oxide synthase (iNOS). Indeolamine-2,3-dioxygenase (IDO). Inducible nitric oxide synthase (NOS2). Gen PTGS. Interleukin-10 (IL-10). Ciclooxygenase 1 (COX-1)- Ciclooxygenase 2 (COX-2).

Respiratory mechanics in mice

In this study, we evaluated the respiratory system mechanics in mice under closed-chest conditions. For each animal, peak values were determined for all parameters across the different experimental conditions (**Figure 3**). Inspiratory capacity (IC) and responses to both single-frequency and broadband frequency forced oscillation perturbations were carefully monitored. The operating software automatically calculated several inspiratory parameters for each perturbation, including resistance (R), elastance (E), Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H). Group averages were computed and expressed as mean ± standard deviation; n = 5. Statistical comparisons using unpaired t-tests revealed no statistically significant differences among the groups.



B

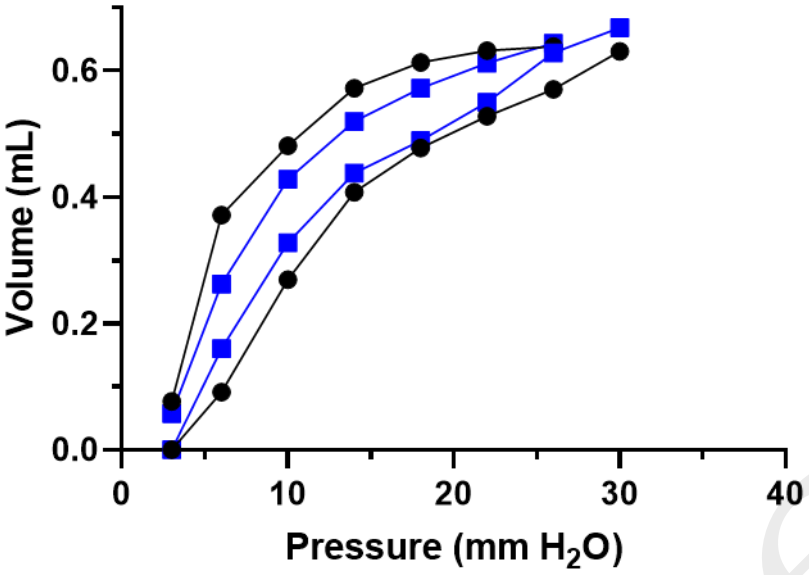
Parameters	Naïve		Exo-Treated	
	Mean	Standard Deviation	Mean	Standard Deviation
IC (mL)	0.606	0.1579	0.750	0.1506
R (cm H_2O.s/mL)	1.507	0.7663	1.514	1.1117
E (cm H_2O/mL)	29.438	6.0479	30.027	15.9204
Rn (cm H_2O.s/mL)	0.498	0.1719	0.443	0.1021
G (cm H_2O/mL)	6.609	2.4555	6.891	3.2547
H (cm H_2O/mL)	21.811	3.2528	24.373	11.4429

Figure 3. Mechanics of the respiratory system. A) Inspiratory capacity (IC) and respiratory parameters were calculated: R (resistance), E (elastance), Rn (Newtonian resistance), G (tissue damping) and H (tissue elastance). B) Results are expressed as the mean ± standard

deviation (n = 5 per group). No significant differences were found between control and treated animals.

Additionally, pressure-volume loops (PVs-P) were generated (**Figure 4A**) to obtain static compliance of the respiratory system, an estimate of inspiratory capacity (A), curvature (K) of the upper portion of the deflation limb of the PV curve, and the area enclosed by the PV loop (Area). Once again, no statistical significative differences were observed between groups (**Figure 4B**).

In summary, these results in our murine model indicate that supernatants derived from equine polarized-MSC are safe and do not induce physiology alterations in the lungs of treated mice.



Parameters	Naïve		Exo-Treated	
	Mean	Standard Deviation	Mean	Standard Deviation
A (mL)	0.795	0.2550	0.691	0.2022
K (/cm H_2O)	0.149	0.0576	0.155	0.0481
C_{st} (ml/cm H_20)	0.061	0.0112	0.068	0.0142
Area (ml.cm H_2O)	1.609	0.2396	1.502	0.3523

Figures 4 A and **B**. Mechanics of the respiratory system. A) Pressure-volume curves in naïve C57BL/6 and exo-treated mice. Curves were generated using a stepwise pressure-driven perturbation (PVs-P) to ensure that each mouse lungs were inflated to the same pressure, independently of their condition. B) Salazar-Knowles equation parameters extracted from individual pressure-volume curves were also averaged and reported in a table format. Results are expressed as the mean ± standard deviation (n = 5 per group). No significant differences were found between control and treated animals.

Inspiratory capacity (A). curvature (K). Static compliance (C_{st})

The first control group of healthy horses (HC) received treatments with polarized MSC supernatant. The second group, consisting of horses diagnosed with asthma, was treated with non-polarized MSC supernatant (ANC). Finally, the third and fourth groups included asthmatic horses treated with polarized-MSC supernatant (**Table 2**). All horses underwent a BAL procedure shortly before the administration of the therapy. Following the application of the MSC supernatant, the animals were closely monitored for any signs of adverse reactions in the hours immediately after treatment. This monitoring was extended over the following days and included routine physical examinations to ensure comprehensive follow-up. Importantly, none of the treated horses exhibited clinical signs suggestive of adverse effects related to the procedures or the therapy (**Table 2**).

Table 2. Characteristics of the horse groups studied

Group	N	Age (years)	Treatment applied	ALpost-treatment
Healthy controls	3	18 ± 4	Supernatant of MSC pre-conditioned with IFN-γ and TNF-α	3 days
Horses with asthma symptoms	2	14.0 ± 1.4	Supernatant of MSC non-pre-conditioned	
Horses with asthma symptoms (ANCP)	3	16.0 ± 3	Supernatant of MSC pre-conditioned with IFN-γ and TNF-α	3 days
Horses with asthma symptoms (ACMP)	4	11.8 ± 4.9	Supernatant of MSC pre-conditioned with IFN-γ and TNF-α	10 days

HC group,: HC group, healthy horses treated with polarized mesenchymal stem cells (MSC). ANCP horses were clinically re-evaluated after 3 days. ACMP horses were clinically re-evaluated after either 10 days.

To assess the cellular response to the therapy, the horses were clinically re-evaluated after either 3 (ANCP) or 10 days (ACMP), depending on their assigned study group, Subsequently, a second bronchoalveolar lavage (BAL) procedure was performed. This evaluation included the clinical analysis of multiple variables (**Table 3**).

Table 3. Clinical results in horses

	Nasal discharge (0-3)		Cough (0-3)		Crepitation (0-2)		Wheezing (0-2)		Exercise intolerance (0-3)		Delay in recovery (0-3)		Total clinical score	
Horses	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
HC 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HC 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HC 3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ANC 1	2	1	3	1	2	2	2	2	2	2	2	0	13	8
ANC 2	2	2	3	3	1	1	1	1	2	2	2	2	11	11
ANCP 1	0	0	2	1	2	1	1	1	1	0	3	1	9	4
ANCP 2	2	1	2	1	0	0	2	0	0	0	0	0	6	2
ANCP 3	1	1	2	1	0	0	2	2	0	0	0	0	5	4
ACMP 1	3	0	2	0	2	2	0	0	3	1	2	1	12	4
ACMP 2	0	0	3	3	1	1	2	2	3	3	2	2	11	11
ACMP 3	0	0	2	0	0	0	2	2	0	0	0	0	4	2
ACMP 4	1	0	2	0	0	0	0	0	1	0	0	0	4	0

Healthy horses group (HC) treated with polarized mesenchymal stem cells (MSC). Asthma horses treated no polarized supernatant (ANC). Asthma horses treated with polarized supernatant (ANCP) horses were clinically re-evaluated after 3 days. Horses with asthma symtoms (ACMP) horses were clinically re-evaluated after 10 days.

The HC group, comprised of healthy horses treated with polarized MSC, showed no significant alterations in bronchoalveolar lavage fluid (BALF) cell counts post-treatment, as shown in **Table 3**. The cell types analyzed included macrophages, lymphocytes, neutrophils, mast cells, and eosinophils. Data presented in **Table 4** reveal that the cell counts remained relatively stable across all control horses, both pre- and post-treatment. Following treatment, the healthy horses did not show a significant change in the percentage of neutrophils in BAL

(median 8 % ± 0.3), whereas the horses with asthma that were treated with supernatant from non-polarized MSC (ANC) exhibited a significant increase to 66.2 % (SE: ± 0.

Table 4. Bronchoalveolar Lavage Fluid in Healthy horses, Asthma horses treated no polarized supernatant and Asthma horses treated with polarized supernatant, (% cells)

		Macrophages	Lymphocytes	Neutrophils	Mast cells	Eosinophils
HC	pretreatment	**39.80**	**43.80**	**8.90**	**1.70**	**5.80**
	posttreatment	**39.23**	**45.23**	**7.74**	**1.40**	**6.40**
ANC	pretreatment	**24.30**	**19.7**	**55.2**	**0.50**	**0.30**
	posttreatment	**29.3**	**23.8**	**44.7**	**1.60**	**0.60**
ANCP	pretreatment	**27.50**	**61.40**	**9.56**	**1.24**	**0.30**
	posttreatment	**28.50**	**60.90**	**8.40**	**1.60**	**0.60**

Healthy horses group (HC) treated with polarized mesenchymal stem cells (MSC). Asthma horses treated no polarized supernatant (ANC) horses were clinically re-evaluated after 3 days. Asthma horses treated with polarized supernatant (ANCP) horses were clinically re-evaluated after either 10 days.

On the other hand, the ANCP group, composed of asthmatic horses treated with polarized supernatant, also did not show significant changes in the percentage of cells in BAL at day 3 after treatment (**Table 3**).

Finally, in the ACMP group, ten days after polarized-MSC treatment, the asthmatic horses showed different clinical signs (**Table 2**) and a distinct BAL pattern (**Table 4**). There was a marked decrease in neutrophil counts and increases in macrophage and lymphocyte values (**Table 5**). These findings suggest a shift in the inflammatory response over time.

Table 5. Bronchoalveolar Lavage Fluid cell counts after 10 days in asthma horses of polarized MSC treatment: ACMP (% cells)

		Macrophages	Lymphocytes	Neutrophils	Mast cell	Eosinophils
ACMP1	pretreatment	**14.2**	**12.4**	**69.2**	**0.5**	**0.3**
	posttreatment	**38.3**	**25.33**	**36.47**	**0.4**	**0.3**
ACMP2	pretreatment	**33**	**14.3**	**18.4**	**0.5**	**0.6**
	posttreatment	**27.7**	**25.3**	**46**	**0.4**	**0.6**
ACMP3	pretreatment	**46.5**	**31.8**	**18.8**	**1.02**	**1.7**
	posttreatment	**62.8**	**29.7**	**6.7**	**0.5**	**0.3**
ACMP4	pretreatment	**61.3**	**22.4**	**13.6**	**1.9**	**0.8**
	posttreatment	**38.6**	**52.1**	**8.03**	**0.5**	**0.8**

HC group, healthy horses treated with polarized mesenchymal stem cells (MSC). ANCP horses were clinically re-evaluated after 3. ACMP horses were clinically re-evaluated after either 10 days.

Discussion

Respiratory pathologies, such as asthma, persist as a significant medical challenge. Despite advancements in conventional treatments, effective long-term management of patients remains elusive. Currently available bronchodilators, corticosteroids, and immunotherapy have demonstrated utility, but side effects and long-term resistance limit their efficacy.[1]

In the search for more specific and less invasive therapies, our group hypothesized that utilizing conditioned medium from MSC cultures could prove to be a safe, effective, and clinically manageable therapy. Collecting the products secreted by MSC could offer an immunomodulatory therapy. Still, based on current knowledge regarding the polarization of these cells, it seemed more appropriate to direct MSC towards a more immunosuppressive phenotype to enhance their therapeutic effect. In this context, various studies have recently been published investigating the polarization of MSC.[15–19] Exposure to pattern recognition system ligands, such as TLRs, has proven effective in inducing the polarization of these cells.[18] Similarly, exposure to proinflammatory cytokines, such as IFN-γ and TNF-α, has been identified as another way to induce polarization towards a more immunosuppressive phenotype.[20] These signals activate intracellular pathways, such as the JAK/STAT signaling pathway, regulating the expression of immunosuppressive effector molecules. Once the cells are polarized, there will be a release of soluble factors, such as PGE2 and IL-10, which together play a crucial role in inhibiting the proliferation and activity of effector cells of the immune system, thus promoting an anti-inflammatory environment.

The utilization of horses as a model for human asthma emerges as an innovative and relevant strategy in the quest for new treatments. Anatomical and physiological similarities between the equine and human respiratory systems support the relevance of this model in understanding and treating respiratory diseases.[2] So, initially, we studied the best way to polarize horse MSC. Interestingly, in our hands, a TLR ligand, such as Poly:IC, could not substantially modify the state of equine MSC, in contrast to what has been published by other authors.[18] On the other hand, exposure to IFN-γ and TNF-α induced an anti-inflammatory phenotype in these equine cells. Cells polarized with IFN-γ and TNF-α showed an increase in the expression of IDO, NOS2, iNOS, IL-6, and COX-2, with statistical significance observed only in the IL-6 and iNOS genes. These results, on the contrary, do coincide with findings published by Caffi et al.[20]

Then we assessed the safety of the conditioned medium derived from polarized cells in preclinical murine studies. The administration of conditioned medium from equine MSC in mice was well-tolerated, with no observed tissue or physiological alterations in lung mechanics. Importantly, this safety was observed in immunocompetent mice, even when using equine conditioned medium, highlighting the biosecurity of such treatments.

We then evaluated the safety of the polarized conditioned medium in our equine model. Initial results for treated horses showed no significant alterations, clinically or in the BALF cell count, after treatment. Cell types analyzed included macrophages, lymphocytes, neutrophils, mast cells, and eosinophils, with cell counts remaining relatively stable in all horses in the control healthy group, both before and after treatment. Subsequently, we decided to treat horses with asthma. Initially, with a clear focus on treatment safety, we treated these diseased

horses with conditioned medium from non-polarized MSC. Previously, it was published that conditioned medium from MSC have a strong capacity to inhibit immune cell proliferation, and priming is not necessary to improve their immunosuppressive activity.[21] In this group, the supernatant from our non-polarized MSC did not induce any therapeutic effect, but nor did it cause any significant side effects, both in clinical signs and in the BALF cell count ten days post-treatment. Finally, we conducted treatments with conditioned medium from polarized MSC in horses with asthmatic pathology. We monitored clinical signs and analyzed BALF three and ten days post-treatment. After ten days post-treatment, the cell counts in the BALF showed a different pattern, with a marked decrease in neutrophil counts and increases in macrophage and lymphocyte values. Regarding the BAL, the results allow for the evaluation of inflammatory exudate behavior, but it is essential to consider the physical and clinical examination to interpret results, aligning with the comments of Michelotto et al.[16] stating, "While there have been significant advances in the study of lung disease in horses over the last two decades and considerable progress in complementary diagnostic methods, it is essential to clarify that these techniques do not replace a complete physical examination." Additionally, they allow for the detection of lung abnormalities, as referred to by Couëtil et al, and Hodgson and Hodgson.[17, 22] In this regard, in our study, we also examined the characteristic clinical signs of asthma, and likewise, an improvement in some parameters such as cough and exercise intolerance was observed in the majority of treated horses. Although clinical results were not statistically analyzed, treated horses showed an improvement in their symptoms through follow-up in the months following.

In sum, while it is necessary to continue expanding the study to confirm this trend, this proof of concept confirms the safety of this type of treatment, both in murine experimental models and in healthy and asthmatic horses. Similarly, there is a trend towards obtaining potential beneficial effects from conditioned medium obtained from MSC polarized towards an anti-inflammatory phenotype.

Conclusions

The results obtained from this study support the safety of conditioned medium from polarized MSC in murine model and equine SEA. The trend to modulate the immune response, coupled with the absence of significant side effects, suggests this conditioned medium, based on the polarization of MSC, as an immunomodulatory therapy in pulmonary diseases. This innovative approach opens exciting perspectives for the treatment of asthma and other respiratory conditions.

Data availability

All relevant data are within the manuscript and its supporting information files.

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Conflicts of interest

The authors declare no conflict of interest.

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