Low levels of microRNA-21 in neutrophil-derived exosomes may contribute to airway smooth muscle hyperproliferation in horses with severe asthma

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OBJECTIVE
Neutrophilic inflammation is associated with the degree of airway obstruction in severe equine asthma (SEA), but the contribution of these leukocytes to bronchial remodeling remains ill defined. Neutrophils could cause structural alterations of the airways by the release of exosomes, a type of cell-derived nanoparticles that can modify the biology of local and distant cells. Neutrophil-derived exosomes have been shown to increase airway smooth muscle (ASM) cell proliferation in humans and horses. Therefore, this study aimed to identify neutrophil exosomal microRNAs (miRs) implicated in the regulation of ASM biology in SEA.

ANIMALS
6 horses with SEA and 6 healthy controls.

METHODS
The expression of selected miRs in exosomes from peripheral neutrophils was studied by quantitative PCR. The effects of miR-21 transfection in ASM cells were evaluated by gene expression analysis and proliferation studies.

RESULTS
The miR-21 was downregulated in neutrophil exosomes from SEA horses, and it attenuated the proliferation of ASM cells stimulated with lipopolysaccharide.

CLINICAL RELEVANCE
The lower level of miR-21 in neutrophil-derived exosomes could contribute to ASM hyperproliferation, which could, in turn, promote the thickening of the bronchial wall in SEA.

Keywords: asthma, horses, microRNA, airway smooth muscle, neutrophil extracellular vesicles

Equine asthma is a heterogeneous disease in which affected animals react excessively to a vast array of inhaled antigens, including fungal spores, pollen, organic dusts, and endotoxins. When horses with severe asthma (SEA) are exposed to those airborne particles, mainly through hay feeding, airflow obstruction, neutrophilic airway inflammation, and bronchial wall remodeling occur. Among the structural alterations of the airways by the release of exosomes, a type of cell-derived nanoparticles that can modify the biology of local and distant cells. Neutrophil-derived exosomes have been shown to increase airway smooth muscle (ASM) cell proliferation in humans and horses. Therefore, this study aimed to identify neutrophil exosomal microRNAs (miRs) implicated in the regulation of ASM biology in SEA.

Similarly, SEA in humans is often coupled with airway neutrophilia, an inflammatory subset associated with several negative clinical outcomes, including persistent fixed airway obstruction. Equine and human asthma similarities include hyperplasia as the main mechanism responsible for ASM thickening. Elucidating pathways involved in ASM proliferation and in the relationships between inflammatory and structural cells could contribute to a better understanding of asthma pathogenesis and to the development of alternative therapies for human and equine patients.

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Neutrophils might act as key components of airway dysfunction through the release of various mediators, including extracellular vesicles (EVs). These nanoscale membrane-derived particles are lipid bilayer structures enriched in proteins, DNA, lipids, and RNA, including microRNA (miR).\textsuperscript{12} miRs are non-coding short single-stranded RNAs that modulate cell differentiation and proliferation by regulating gene expression posttranscriptionally through the degradation of mRNA or repression of translation.\textsuperscript{12} Consequently, the internalization of neutrophil EVs and their functional cargo by airway recipient cells could contribute to airway remodeling, including extracellular matrix destruction\textsuperscript{13} and, importantly, ASM proliferation.\textsuperscript{14–16} Therefore, the expression of miR related to asthma pathogenesis and smooth muscle remodeling\textsuperscript{17} was studied in neutrophil-derived exosomes, the smallest form of EVs, collected from horses affected by SEA and healthy animals. More precisely, miRs previously shown to alter ASM biology, including cellular proliferation and production of inflammatory molecules\textsuperscript{18–24} were specifically selected. The objectives of this study were to evaluate the expression of these miRs from neutrophil-derived exosomes in SEA and to determine if differentially expressed miRs could modulate ASM biology. Exploring the roles of exosomal miR is warranted as these molecules have the potential to serve as biomarkers for asthma diagnosis and represent a hope for future targeted biological therapy.

**Methods**

**Animals, selection criteria, and exposure protocol**

All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (Rech-1324) on October 11, 2016. The datasets generated in the current study are available in the UdeM Dataverse repository, (https://doi.org/10.5683/SP3/9AANYK).

Twelve adult horses (10 mares and 2 castrated males) were studied, including 6 horses with SEA that were part of a research herd and 6 healthy mares that were part of the Université de Montréal teaching herd. The horses affected by SEA had a history of recurrent episodes of respiratory distress upon natural antigenic exposure (hay feeding and stabling), and their disease was confirmed before the inclusion in the research herd by assessing the development of airway obstruction (pulmonary resistance > 1 cm H₂O/L/s) and pulmonary inflammation (increased proportion of neutrophils ≥ 25%) in bronchoalveolar lavage fluid) after exposure to hay.\textsuperscript{25} The control horses had no history of respiratory disease despite hay being the primary component of their diet and did not develop airway neutrophilia or abnormal lung function when stabled and fed hay in previous experiments. Before sampling collection, all horses were antigen exposed by being stabled and fed hay for 1 month, as these conditions are consistent triggers of disease exacerbation in asthmatic horses. Part of the lung function data from horses with SEA had been previously published\textsuperscript{26} as these horses were included in a randomized controlled clinical trial after the antigenic exposure. The control mares remained free of respiratory signs, but lung function and bronchoalveolar lavage fluid data were not collected in this study.

**Neutrophil isolation and culture**

After a 40-minute sedimentation of EDTA anti-coagulated blood (160 mL/horse) at room temperature, the plasma-rich layer was collected for isolation of peripheral blood neutrophils by density gradient centrifugation using Ficoll-Paque (Ficoll-Paque PREMIUM 1084; GE Healthcare Bio-Sciences Corp) according to the manufacturer’s instructions and as previously described.\textsuperscript{14,16} Cells were counted and viability was assessed using a fluorescence automatic cell counter (ADAM Cell Counter; Montreal-Biotech Inc). Cytospin slides were prepared and stained with Protocol Hema 3 (Fisher) for differential counting of 400 cells to assess neutrophil purity. The purity and viability of neutrophils were 99.1 ± 0.8% and 98.2 ± 0.7%, respectively (mean ± SD), and were deemed adequate to pursue cell culture. Neutrophils (3 X 10⁶ cells/condition) were suspended at 5 X 10⁶ cells/mL in the RPMI 1640 culture medium supplemented with 10% heat-inactivated low-endotoxin and EV-free FBS, 2 mM l-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and cultured in 6-well plates (nontreated plastic) for 18 hours in a CO₂ incubator at 37°C. Supernatants were then kept at −20°C until the isolation of RNA in exosomal extracts (ExoRNA).

**ExoRNA isolation**

The RNA from neutrophil exosomes was extracted from the culture supernatant (25 mL) using membrane affinity columns. The first step of centrifugation was performed at 3,000 g with the Centricron Centrifugal Filter (Millipore; 10 KDa) device and then at 4,000 X g for 25 minutes to concentrate the preparation before exoRNA isolation with the exoRNeasy Kit (Qiagen). The concentration and purity of RNA were evaluated by spectrophotometry (all samples with the 260/280 absorbance ratio > 1.8 and mean and SEM of 2.1 ± 0.1).

**miR screening**

This investigation focused on the differential regulation of exosomal miRs previously shown to be involved in the biology of ASM cells and in asthma. More specifically, miRs implicated in the modulation of ASM proliferation (miR-21,\textsuperscript{18,19} miR-143,\textsuperscript{20} and miR-145\textsuperscript{21}), contraction (miR-25\textsuperscript{22} and miR-133\textsuperscript{27}), hypertrophy (miR-26\textsuperscript{28}), migration (miR-21\textsuperscript{18} and miR-145\textsuperscript{21}), gene expression of proinflammatory mediators (miR-25,\textsuperscript{22} miR-146,\textsuperscript{25} and miR-155\textsuperscript{25}) and myofibroblast differentiation (let-7\textsuperscript{29}) were studied. Reverse transcription and quantitative PCR (qPCR) were performed using TaqMan MicroRNA Assays (Invitrogen) designed specifically for each miR of interest following the manufacturer’s instructions (Table 1). Assays were based on human miR
sequences, as they were identical to those found in horses (miRBase Sequence Database and Registry; miRBase.org; accessed in November 2016).30

Quantitative PCR was performed by monitoring the increase of fluorescence of FAM dye in real time with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The relative amount of target miR was calculated using the comparative delta cycle threshold (ΔCt) method, with the CFX Manager Software (Bio-Rad) using miR-199a as the reference miR, as its expression was stable in the various samples (in the neutrophil exosomes of asthmatic and control horses). The Ct is the number of cycles required for the fluorescence to reach a specific level of detection and is negatively correlated with the amount of nucleic acid present in the sample. In the ΔCt normalization method, the Ct of the reference gene is subtracted from the Ct of the tested gene within each sample; thus, a lower value represents a higher expression of the gene of interest. All qPCR reactions were performed in duplicate.

Only miR-21 was differentially expressed in horses with SEA compared to controls. Therefore, specific effects of this miR on ASM biology were evaluated in the next experiments, namely the alteration of selected gene expression18 and cellular proliferation.18,19

**ASM cell isolation**

Bronchial tissues were collected from horses’ lungs from a slaughterhouse where death was caused by a gunshot to the head followed by exsanguination. ASM cells were isolated by enzymatic digestion of bronchial tissue, as previously described.32 Experiments were conducted with 6 different lung donors.

**miR-21 transfection**

The miRs were synthesized by Thermo Fisher Scientific Inc (ID miR-21: 000397; miR Mimic Negative Control: 4464058). Isolated ASM cells (0.25 X 10^6 for 24-well plate or 14,000 cells for proliferation assay) were first transfected with 30 pmol of each miR using Hiperfect reagent (Qiagen) according to the manufacturer’s recommendations. In some experiments, ASM cells were stimulated with lipopolysaccharide (LPS) as inhalation of this omnipresent bacterial fragment leads to airway obstruction and contributes to neutrophilic inflammation in both human and equine asthmatics.1,35-37 Cell extracts were kept in Trizol Reagent (Invitrogen) until RNA extraction.

**ASM gene expression analysis**

The expression of selected genes previously shown to be modulated by miR-21 and potentially involved in smooth muscle remodeling was evaluated by qPCR. More specifically, the expression of the proinflammatory cytokine C-X-C chemokine ligand 8 (CXCL-8) and of the major inflammatory transcription factor nuclear factor-kappa B (NF-κB) were evaluated as both are regulated by miR-2136,37 and induce ASM proliferation.38,39 The expression of 2 tumor-suppressor genes also regulated by miR-21 was studied due to their reported association with cell-cycle regulation and asthma [programmed cell death 4 (PDCD4)40-42 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN)].18,42 Total RNA extraction was performed on transfected cell extracts prepared using the RNeasy Mini Kit (Qiagen). Three hundred nanograms of total RNA was reverse transcribed using 200 U/μL Superscript III with 40 U/μL RNaseOUT Recombinant Ribonuclease Inhibitor and Oligo(dt) Primer (final concentration of 2.5 μM; all from Invitrogen). Samples were reverse transcribed and purified in duplicate. Quantitative PCR was performed using theRotor-Gene RG3000 (Corbett Research) with the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer’s instructions, with minor modifications. Briefly, 2 μL of cDNA template was added to obtain a 20-μL final reaction volume containing 0.5 μM forward and reverse primers. Amplification conditions included a denaturation step of 10 minutes at 95 °C followed by 45 cycles of denaturation, annealing and elongation steps, and 1 melting curve. The concentration of each target gene cDNA was calculated relative to its standard curve and then normalized with the reference gene ribosomal protein L9. Primers were designed based on the predicted equine sequence to span exon-intron boundaries and to prevent amplification of genomic DNA (Table 2). PDCD4 primers were designed to amplify the 4 predicted equine mRNA isoforms. Each primer set generated only 1 PCR product.

**Table 1—Sequences of microRNA assays in equine neutrophil exosomes.**

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Sequence 5’–3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-21-5p</td>
<td>UAGCUUAAUCAUGACUGAAGUGU</td>
</tr>
<tr>
<td>hsa-miR-25-3p</td>
<td>CAUUGCAUCUGUCUGCGGUGA</td>
</tr>
<tr>
<td>hsa-miR-26-5p</td>
<td>UUCAAGAAUCAUGACUGUAGGCU</td>
</tr>
<tr>
<td>hsa-miR-133a-3p</td>
<td>UUUGGGUCCCUUCAACAGCGUG</td>
</tr>
<tr>
<td>hsa-miR-143-3p</td>
<td>UGAGAUGAAGACUGUAGACUC</td>
</tr>
<tr>
<td>hsa-miR-145-5p</td>
<td>GCUGCAUUUCCCCAGGAUCCCU</td>
</tr>
<tr>
<td>hsa-miR-146a-5p</td>
<td>UGAGAAGCGAUAUUCCAUUUGGU</td>
</tr>
<tr>
<td>hsa-miR-155-5p</td>
<td>UUAUUGCJUAUUGCUGUAAGGGGU</td>
</tr>
<tr>
<td>let-7-family</td>
<td>CACCGAUUCAGACUCACCUU</td>
</tr>
<tr>
<td>hsa-miR-199a-5p</td>
<td>CCCAGUGUUCAGACUCACCUU</td>
</tr>
</tbody>
</table>

**Table 2—Sequences of primer pairs used for the airway smooth muscle gene expression analysis.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’–3’)</th>
</tr>
</thead>
</table>
| PDCD4 | F: GCCAGCTCTAGCCTGTACA  
R: CCTCTAGAGCACCACAGTT |
| PTEN | F: CTTTGAATTCCCTACGCTGAG  
R: AGGTTCCTCTGGATGTTG |
| NF-κB | F: ACTTTGAAGGCTGAGACTGTTG  
R: CCAGCAAGATCTGAGCACCTC |
| CXCL-8 | F: CCTTCTGCAGCTTGTGTGAAG  
R: GCAGACCTCAGTCTGCTGAC |
| RPL9 | F: TCTAGATATCTCCGCTGCG  
R: TGCACCTCGGAGAACCTC |

Figure 1—MicroRNA (miR) expression in neutrophil-derived exosomes is illustrated with the comparative delta cycle threshold (ΔCt) method. A–I—miR-21 (A), miR-146a (B), miR-26a (C), miR-133a (D), miR-145 (E), let-7 (F), miR-25 (G), miR-143 (H), and miR-155 (I). Data are shown as dot plots with the mean (or median if not normally distributed).
and the identity and integrity of these products were confirmed by sequencing. Samples were run in duplicate with negative controls.

**Smooth muscle proliferation assay**

Proliferation of the transfected ASM cells was monitored in real time by electrical impedance using the xCELLigence RTCA-DP instrument and E-Plates 16 (ACEA Biosciences Inc), as previously reported. Briefly, gold electrodes at the bottom of each well measure the electrical impedance generated from cell proliferation, which is reported as a cell index (CI). The ASM cells were first grown at 37 °C in a humidified atmosphere with 5% CO₂ and monitored for 24 hours to assess baseline cell proliferation. Cells were then transfected with miR-21 or the Mimic Negative Control 24 hours after the ASM were seeded. LPS was added in some wells as described above 24 hours later. At the end of the culture period (72 hours), the final CI was normalized to the last CI recorded before the time of cell transfection. The rate of cell growth was represented by the slope of the line between the time before cell transfection until 24 hours after the LPS treatment (24 to 72 hours), which was calculated by the XCELLigence software (version 1.2.1; Agilent).

**Statistical analysis**

Normality of the data was analyzed with Shapiro-Wilk tests and QQ plot visualization. Data were analyzed with unpaired t test (or Mann-Whitney tests if not normally distributed) for miR expression analysis between asthmatic and healthy horses and with paired (or Wilcoxon tests if not normally distributed) for proliferation analysis and for gene expression studies of ASM cells. Statistical analyses were performed using GraphPad Prism software (version 10.1.0). Differences were considered statistically significant at $P < .05$.

**Results**

**Clinical information**

The 6 horses affected with SEA were aged 13.5 ± 3.2 years and weighed 510 ± 57 kg, while the 6 healthy mares were aged 13.5 ± 4.7 years and weighed 518 ± 34 kg (no difference between groups). After the antigenic exposure, all horses with SEA had pulmonary resistance above 1 cm H₂O/L/s (mean of 2.2 ± 1.2 cm H₂O/L/s), confirming the development of an asthma exacerbation.

**Neutrophil exosomal miR content**

All selected miRs were detected in exosomal extracts, but only miR-21 was differentially expressed, with a decreased expression in neutrophil exosomes from asthmatic animals compared to controls (Figure 1).

**ASM cell gene expression**

The miR-21 transfection into ASM cells did not significantly modify the gene expression of CXCL-8, NF-κB, PTEN, and PDCD4 in unstimulated and LPS-treated ASM cells (Figure 2).
**ASM cell proliferation**

To study the possible contribution of miR-21 to bronchial smooth muscle cell hyperplasia, ASM cell proliferation was assessed in unstimulated and LPS-treated cells. The proliferation of ASM cells transfected with miR-21 was inhibited in the LPS-stimulated condition \( (P < .05, \text{Figure 3}) \), but not in unstimulated ASM cells.

![Figure 3—Airway smooth muscle cell proliferation. Data are presented as a dot plot with the mean of different cultures \( (n = 3; \) each symbol represents a different culture) run in duplicate. LPS = Lipopolysaccharide. miR = MicroRNA.](image)

**Discussion**

This investigation focused on the differential regulation of exosomal miRs previously shown to be involved in the biology of ASM cells and in asthma in other species. Within the selected miRs, only miR-21 was differentially expressed as it was downregulated in neutrophil exosomes of horses affected by SEA compared to controls. The transfection of miR-21 decreased ASM proliferation of cells stimulated by LPS, suggesting that the low miR-21 level in neutrophil exosomes from asthmatic horses might have a role in promoting ASM hyperproliferation.

Although the miR-21 expression in exosomes of peripheral neutrophils has not been specifically investigated in humans with asthma, there are conflicting reports of its expression in plasma and in pulmonary fluid. Most studies have reported an upregulation of circulating miR-21 in the plasma of asthmatic children and adults, with levels correlating with poor lung function and with the severity of the disease. Others observed that miR-21 expression was upregulated in the serum exosomes of moderate to severe asthmatics compared to mild asthmatics but that it was lower in the patients with elevated levels of IL-6, a cytokine associated with higher blood neutrophilia. On the contrary, a lower expression of miR-21 in pulmonary fluid exosomes from human asthmatic patients was reported, which is consistent with the downregulation of miR-21 in the peripheral neutrophils in SEA in the current study. Studying the exosomes from equine airway neutrophils would have been relevant as endothelial transmigration of these leukocytes might modify their biological functions, and perhaps their cellular content in EVs, but thus far, the isolation of neutrophils from the bronchoalveolar lavage fluid does not reach sufficient purity in the equine species.

The expression of miR-21 has also been investigated in ASM cells, where it was upregulated in both humans and horses with asthma. The overall biologic implication of this reported miR-21 overexpression in ASM and the downregulation in neutrophil-derived exosomes in the current study is uncertain. Such an equilibrium may prevent uncontrolled ASM cell hyperproliferation, which could lead to airway occlusion in asthma. Contrary to the decreasing ASM proliferation associated with miR-21 in the current study in horses, the overexpression of miR-21 in human ASM cells leads to hyperproliferation. This finding suggests that the mRNA target of miR-21 might differ between humans and horses and that its effects cannot be directly compared between these 2 species.

ASM cells were studied quiescent and while stimulated with LPS as it contributes to the development of asthma exacerbation in both humans and equine asthmatics, although it is not a major trigger of the disease on its own. In the current study, the reduced proliferation induced by miR-21 only observed in LPS-stimulated ASM cells is more likely a reflection of low power to detect the same change in quiescent cells rather than a more potent effect of miR-21 in stimulated ASM cells. Indeed, LPS stimulation did not increase equine ASM proliferation in the current experiments, which is consistent with previous results. Exploring the effects of miR-21 transfection on equine ASM stimulated with mitogens present in neutrophilic asthma, such as transforming growth factor-β, interleukin-17, and CXCL-8, would be relevant in future studies.

The main limitation of this study is the low number of horses included and cell experiments performed. This could have prevented the detection of a statistically significant effect of LPS on the regulation of CXCL-8 and NF-κB, as it was increased in all cell cultures, as expected based on the literature, but not significantly. Furthermore, the role of miR-21 on other asthma remodeling features was not evaluated, such as its possible effect on muscle contractility, epithelial-to-mesenchymal transition, and pathological fibrosis. Finally, this study evaluated the effects of miR-21 transfection in ASM obtained from nonasthmatic animals. Determining if its actions differ in asthmatic ASM, in which miR-21 is upregulated, would be relevant in future investigations.

In conclusion, the downregulation of miR-21 in neutrophil-derived exosomes from asthmatic horses exposed to an antigenic environment could contribute to ASM proliferation in this species.

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None reported.
Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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