Cytokine mRNA expressions after racing at a high altitude and at sea level in horses with exercise-induced pulmonary hemorrhage

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**Objective**—To determine concentrations of cytokine mRNA in horses with exercise-induced pulmonary hemorrhage (EIPH) after racing.

**Animals**—97 Thoroughbreds.

**Procedures**—Following tracheobronchoscopy, the severity of EIPH was graded (scale of 0 to 4), and venous blood samples were collected from 10 horses in each grade. After RNA isolation and cDNA synthesis, real-time PCR assay was conducted to detect cytokine-specific mRNA for interleukin (IL)-1, IL-6, and IL-10; interferon (INF)-γ; and tumor necrosis factor (TNF)-α.

**Results**—Neither location nor grade of EIPH affected the expression of IL-1 and INF-γ. There was significantly greater overall expression of IL-6 mRNA at sea level, with significantly more IL-6 expressed in horses with grade 4 EIPH than in horses with grade 0, 1, or 2 EIPH. At a high altitude, no difference was detected for IL-6 expression among the various EIPH grades. There was significantly greater overall expression of TNF-α mRNA at a high altitude; however, there was no difference within the various grades of EIPH. Expression of IL-10 was significantly affected by grade of EIPH because horses with grade 3 EIPH expressed significantly more IL-10 mRNA than did horses with grade 0 or 2 EIPH; this expression was not affected by location.

**Conclusions and Clinical Relevance**—At sea level, increased IL-6 expression was associated with more severe EIPH, and altitude may affect gene expressions of the proinflammatory cytokine TNF-α and anti-inflammatory cytokine IL-6. Studies on protein concentrations of cytokine expression are needed. The pathophysiologic importance of these findings remains to be explained. (Am J Vet Res 2010;71:447–453)

Exercise-induced pulmonary hemorrhage is common in racing Thoroughbreds undergoing strenuous exercise, with a reported prevalence of 43% to 75.4%. No precise mechanism has been identified that can account for the site of hemorrhage and progression of EIPH within the lungs; however, pulmonary hypertension with secondary stress failure of pulmonary capillaries has been implicated. Exercise-induced pulmonary hemorrhage is definitively diagnosed by use of postexercise endoscopic examination of the pharynx, larynx, trachea, or mainstem bronchi for the presence and severity of hemorrhage. Examination of tracheal aspirates may reveal RBCs and hemosiderophages, whereas examination of bronchoalveolar lavage fluid may be used to quantify EIPH by measurement of the RBC concentration.

Pulmonary inflammation is often detected in horses with EIPH. This may be attributable to preexisting disease of the small airways or because of blood in the airways.

Furthermore, the local response to tissue injury following stress failure of the pulmonary capillaries may

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**Abbreviations**

- EIPH: Exercise-induced pulmonary hemorrhage
- IL: Interleukin
- INF: Interferon
- TNF: Tumor necrosis factor

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involve the production of cytokines at the site of inflammation, which may be accompanied by a systemic acute-phase response. Because airway inflammation involves the production of such inflammatory mediators, we hypothesized that racing Thoroughbreds with more severe grades of EIPH would have greater systemic expression of cytokine mRNA.

Although, to our knowledge, proinflammatory responses have not been reported before in horses with EIPH, information exists on increased mRNA expression of IL-1β, IL-8, and TNF-α in the bronchoalveolar lavage fluid of horses with recurrent airway obstruction; increased mucosal IL-4 and IL-10 expression associated with Cyathostominae larvae in the wall of the equine large colon; and increased IL-1β, IL-8, and TNF-α expression in leukocytes isolated from blood of horses following infection with Anaplasma phagocytophilum. We chose to study proinflammatory cytokines IL-1, IL-6, and TNF-α, which are cytokines responsible for the induction of fever, neutrophil recruitment, tissue remodeling, and immune activation, and INF-γ, which is a pleotropic cytokine with proinflammatory properties that augments TNF activity. Interleukin-10 was assessed for its potent anti-inflammatory activity because it may suppress proinflammatory cytokines, such as IL-1 and TNF-α.

Because the determination of an association between EIPH and inflammation at a molecular level may assist in the development of preventive strategies aimed at reducing the prevalence and severity of EIPH, the purpose of the study reported here was to measure IL-1, IL-6, IL-10, INF-γ, and TNF-α gene expression in a population of horses with EIPH immediately after racing at sea level and at a high altitude in a racing jurisdiction that did not permit administration of furosemide nor use of nasal dilator strips on race day.

Materials and Methods

Animals—Thoroughbreds of either sex that raced on turf or sand on flat racecourses were enrolled in the study between August and December 2005. The Thoroughbreds competed at 2 racecourses located at a high altitude (> 1,400 m above sea level, Turffontein Race Course [Gauteng Province] and Vaal Race Course [Free State Province]) and at 3 racecourses located at sea level (Clairwood and Greyville Turf Club [Kwazulu-Natal Province] and Kenilworth Race Course [Western Cape Province]) in South Africa. Administration of medications (such as furosemide) was not allowed on race day, and drug testing was strictly enforced by the National Horse Racing Authority through screening of urine and blood samples for the detection of prohibited and therapeutic substances. Lists of available horses that were accepted to race were obtained from the National Horse Racing Authority. Eligible horses were then identified, trainers were contacted, and permission was requested to examine the horses and collect blood samples. Only horses enrolled prior to race day were entered into the cross-sectional study to avoid a potential enrollment bias. The study was approved by the Animal Use and Care Committee and Veterinary Research Committee of the University of Pretoria.

Tracheobronchoscopy and sample collection—Tracheobronchosscopic evaluations were performed to detect evidence of EIPH on 97 unsedated horses within 2 hours after the horses had completed a race. Horses ranged from 3 to 8 years of age (median, 4 years) and had completed 0 to 81 lifetime starts (median, 11 lifetime starts). Tracheobronchoscopic evaluations were performed by use of an endoscope that was passed through one of the nares, the nasopharynx, and the larynx to the level of the tracheal bifurcation. Severity of EIPH was immediately graded by one of the investigators (MNS), who used an established grading system. Grades were assigned from 0 to 4 as follows: grade 0 = no blood in the pharynx, larynx, trachea, or mainstem bronchi; grade 1 = 1 or more flecks of blood or ≤ 2 short (< 25% of the length of the trachea), narrow (< 10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi; grade 2 = 1 long (> 50% of the length of the trachea) or > 2 short (< 25% of the length of the trachea), narrow (< 10% of the tracheal circumference) streams of blood covering < 33% of the tracheal circumference; grade 3 = multiple distinct streams of blood covering > 33% of the tracheal circumference without blood pooling at the thoracic inlet; and grade 4 = multiple coalescing streams of blood covering > 90% of the tracheal surface with blood pooling at the thoracic inlet.

To allow greater sample distribution, identifying a horse with grade 3 or 4 EIPH initiated a specific sample collection routine. Venous blood samples (2.5 mL) were collected by use of routine jugular venipuncture from horses with grade 3 EIPH (followed by 1 horse each with grade 0, 1, and 2 EIPH) and grade 4 EIPH (followed by 1 horse each with grade 0, 1, 2, and 3 EIPH). Blood samples were collected directly into RNA collection tubes within 2 hours after racing. The sample collection routine was repeated until samples were obtained from 10 horses in each EIPH grade classification (0 to 4) at both a high altitude and at sea level, except for horses with grade 4 EIPH at a high altitude for which only 7 horses were identified. Immediately after blood samples were collected, each tube was inverted 10 times to prevent coagulation that would hinder future RNA extraction. The tubes were maintained at 25°C overnight and then stored at −20°C until RNA extraction was performed.

RNA extraction and cDNA synthesis—Samples were thawed. Cell pellets were obtained by centrifugation at 2,500 × g, and RNA was extracted in accordance with the manufacturer’s protocol, with slight modifications. Briefly, cell pellets were treated with cell lysis buffer containing proteinase K for 5 minutes at 25°C. Samples then were heated at 55°C for 10 minutes, which was followed by centrifugation for 10 minutes. Total RNA was eluted in 40 µL of RNase-free water and stored at −80°C. The cDNA was synthesized in accordance with the manufacturer’s protocol.

Real-time PCR assay—Real-time PCR assay was performed by use of an automated system. The real-time PCR reaction mixtures had a final volume of 10 µL/well, which consisted of 4.5 µL of the synthesized cDNA strand, 5 µL of Taq polymerase, and 0.5 µL of primers and probes. Water was included in wells on each plate as a negative control sample. Amplification
Nonparametric tests were provided in a kit that contained both the designed primer and probe in solution (Appendix). To allow for potential variability in sample processing, expression of the genes of interest was initially compared with expression of β-glucuronidase. This control gene has the lowest variability, compared with that of glyceraldehyde-3-phosphate dehydrogenase. Additionally, relative quantitation of gene expression was performed in accordance with the method of Livak and Schmittgen, in which the internal calibrator used was the mean of the value for grade 0 EIPH samples. Each cDNA sample was amplified in duplicate, and all reaction solutions and samples were added to the plate via a robotic pipetting machine to ensure that study samples were most accurately and reproducibly pipetted. The end point threshold cycle (ie, C$_R$) was defined as the PCR cycle number that crossed the signal threshold; it ranged from 0 (no product) to 40.

Statistical analysis—Nonparametric tests were used to compare overall differences in target gene expression within grades of EIPH (Spearman rank-order correlation) because the data were not normally distributed, with post hoc analyses performed by use of the Holm-Sidak t test. Linear regression was used for comparisons between location (high altitude vs sea level) and EIPH grade. Significance was set at values of $P < 0.05$. Statistical tests were conducted by use of commercially available computer software.

Results

Cytokine mRNA expression was determined in 10 horses that raced at a high altitude for each EIPH grade classification, except for grade 4 EIPH in which only 7 horses were identified. This represented 4.7% (10/211), 8.3% (10/120), 22.7% (10/44), 38.5% (10/26), and 100% (7/7) of the horses with an EIPH grade of 0, 1, 2, 3, and 4, respectively. Cytokine mRNA expression was determined in 10 horses that raced at sea level for each EIPH grade, which represented 4.0% (10/251), 8.3% (10/120), 22.7% (10/44), 38.5% (10/26), and 100% (7/7) of the horses with an EIPH grade of 0 through 4, respectively. Mean expression of IL-1, IL-6, IL-10, INF-γ, and TNF-α mRNA was determined (Figures 1–5).

Neither location of the racecourse nor EIPH grade significantly affected expression of IL-1 or INF-γ, although there was a slight increase in expression with severe hemorrhage in both cytokines for horses that raced at sea level (Figures 1 and 4). There was significantly ($P = 0.01$) greater overall expression of IL-6 mRNA in horses that raced at sea level than in horses that raced at a high altitude, with significantly ($P = 0.01$) more IL-6 expressed in racehorses with grade 0 EIPH, compared with IL-6 expression in horses with grade 0, 1, and 2 EIPH (Figure 2). The IL-6 expression did not differ significantly ($P = 0.05$) among the Thoroughbreds with grade 0 to 4 EIPH after racing at racecourses located at a high altitude (> 1,400 m above sea level [black bars]) and at sea level (white bars). Grades were assigned from 0 to 4 as follows: grade 0 = no blood in the pharynx, larynx, trachea, or mainstem bronchi; grade 1 = 1 or more flecks of blood or ≤ 2 short (< 25% of the length of the trachea), narrow (< 10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi; grade 2 = 1 long (> 50% of the length of the trachea) or > 2 short streams of blood covering > 33% of the tracheal circumference; grade 3 = multiple distinct streams of blood covering > 33% of the tracheal circumference without blood pooling at the thoracic inlet; and grade 4 = multiple coalescing streams of blood covering > 90% of the tracheal surface with blood pooling at the thoracic inlet. Expression of IL-1 mRNA did not differ significantly ($P ≥ 0.05$) among EIPH grades or between locations.

Figure 1—Mean ± SD relative expression of IL-1 mRNA in 97 Thoroughbreds with grade 0 to 4 EIPH after racing at racecourses located at a high altitude (> 1,400 m above sea level [black bars]) and at sea level (white bars). Grades were assigned from 0 to 4 as follows: grade 0 = no blood in the pharynx, larynx, trachea, or mainstem bronchi; grade 1 = 1 or more flecks of blood or ≤ 2 short (< 25% of the length of the trachea), narrow (< 10% of the tracheal surface area) streams of blood in the trachea or mainstem bronchi; grade 2 = 1 long (> 50% of the length of the trachea) or > 2 short streams of blood covering > 33% of the tracheal circumference; grade 3 = multiple distinct streams of blood covering > 33% of the tracheal circumference without blood pooling at the thoracic inlet; and grade 4 = multiple coalescing streams of blood covering > 90% of the tracheal surface with blood pooling at the thoracic inlet. Expression of IL-1 mRNA did not differ significantly ($P ≥ 0.05$) among EIPH grades or between locations.

Figure 2—Mean ± SD relative expression of IL-6 mRNA in 97 Thoroughbreds with grade 0 to 4 EIPH after racing at racecourses located at a high altitude and at sea level. Expression of IL-6 mRNA in horses racing at sea level differed significantly ($P < 0.05$) from expression in horses racing at a high altitude. *Expression of IL-6 mRNA in horses with grade 4 EIPH differed significantly ($P < 0.05$) from expression for horses with grade 0, 1, or 2 EIPH. See Figure 1 for remainder of key.

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various grades of EIPH for horses that raced at a high altitude. There was significantly ($P = 0.01$) greater overall expression of TNF-α mRNA for horses that raced at a high altitude, compared with expression for horses that raced at sea level; however, TNF-α expression did not differ significantly ($P = 0.06$) among EIPH grades or between locations. See Figure 1 for remainder of key.

Discussion

Pulmonary inflammation in horses with more severe forms of EIPH is associated with histologic evidence of small airway disease and inflammation in bronchoalveolar lavage fluid and tracheal aspirates. Moreover, autologous intrapulmonary blood inoculation in horses causes prolonged airway inflammation. Whether inflammation is a direct consequence of EIPH or whether the inflammation predisposes a horse to EIPH is unknown. Nevertheless, stress failure of the pulmonary capillaries with resultant tissue injury may cause an intrapulmonary upregulation of proinflammatory cytokines, which may be accompanied systemically by an acute-phase response.

In the study reported here, expression of IL-1, IL-6, IL-10, INF-γ, and TNF-α was determined in a population of Thoroughbreds with various grades of EIPH that competed at racecourses located at different altitudes. Availability of monoclonal or polyclonal antibodies for those cytokines would have made comparison of mRNA and protein concentrations possible, although we assumed that mRNA concentrations reflected those of the biologically active cytokines. Furthermore, several studies have revealed a good correlation between inflammatory cytokine gene expression and disease conditions in horses. An increase in mRNA expression of IL-4, TNF-α, and IL-10 mRNA was detected in leukocytes isolated from blood 6 hours after lipopolysaccharide inhalation, whereas there were no change in IL-4, IL-5, IL-13, and IFN-γ mRNA expression.
in lymphocytes isolated from blood and bronchoalveolar lavage fluid of horses with recurrent airway obstruction.6,7

In another study8 conducted by our laboratory group, we described the effect of altitude on the prevalence and severity of EIPH in racing Thoroughbreds in South Africa as determined via tracheobronchoscopy and concluded that EIPH is more prevalent and more severe in horses that race at sea level. This is certainly surprising because plausible reasons exist as to why there may be a greater prevalence and increased severity of EIPH in horses that race at a high altitude. Strenuously exercised racing horses often have exercised-induced arterial hypoxemia9,10 and develop pulmonary arterial hypertension11 that leads to pulmonary capillary stress failure.12 High altitude–induced hypoxic vasoconstriction may worsen the degree of exercise-induced arterial hypoxemia,13 which may directly cause EIPH or may worsen preexisting EIPH. Further research is clearly needed to establish the reason that the prevalence and severity of EIPH are greater in horses that race at sea level.

Exercise-induced pulmonary hemorrhage may be quickly and easily assessed by use of tracheobronchoscopic examination because this technique is minimally invasive and allows immediate grading of racing horses with EIPH, without laborious, time-consuming processing of samples in a laboratory. Although the repeatability of the tracheobronchoscopic grading system used in the present study has been established,14 the relationship between the volume of blood in the airways and actual hemorrhage is not known. In the study reported here, the authors speculated that horses with higher grades of EIPH may have had more hemorrhage.

Although no significant effect of exercise on IL-4, IL-12, and IFN-γ mRNA expression was detected in another study,15 the study reported here is, to the authors’ knowledge, the first in which an association between mRNA expression and EIPH has been reported. It is tempting to speculate that racing horses with a higher grade of EIPH and more blood loss had greater anti-inflammatory IL-6 mRNA expression, which may suggest the activation of an anti-inflammatory mechanism to restrict the magnitude of the inflammatory response. Location of the racecourse appeared to affect overall mRNA expression because more IL-6 was expressed in horses that raced at sea level, whereas greater TNF-α expression was evident in horses that raced at a high altitude.

Stressors such as hypoxia or exercise may initiate an immune and inflammatory response16 characterized by increased expression of IL-6 and TNF-α. In humans, exercise following acute exposure to high altitude is associated with an increase in IL-6 expression but not an increase in TNF-α expression,17,18 whereas TNF-α expression is elevated after prolonged and intense exercise at sea level.19 The study reported here differs from those other studies because IL-6 expression was increased in horses that raced at sea level and TNF-α expression was greater in horses that raced at a high altitude. Because horses raced over shorter distances at sea level, it is possible that overexertion may have further increased transmural pressures of pulmonary capillaries and led to a more profound increase in IL-6 expression, which was counterbalanced by upregulation of anti-inflammatory IL-10 expression in horses with more severe grades of EIPH. Moreover, at a high altitude, horses competing over longer distances may have expressed more TNF-α mRNA, which is similar to results found in human athletes.20

Altitude and EIPH grade had no effect on IL-1 or INF-γ mRNA expression. Studies21,22 in humans have also found only minor or no change in IL-1 mRNA expression after exercise; this may be attributable to rapid systemic clearance because IL-1 mRNA was found in muscle biopsy specimens collected after strenuous exercise without an increase in systemic IL-1 concentrations23 and because of IL-1 concentrations in the urine of runners.24 Furthermore, INF-γ assists in immunomodulation as well as in recruitment and activation of lymphocytes and also has antipathogen activity.25 Because an infectious cause has not been implicated in the pathogenesis of EIPH, it is not surprising that INF-γ affecting cell-mediated cytotoxicosis consistently had low expression in the racing horses, regardless of EIPH grade or racecourse location. However, despite a lack of significant differences, racing horses with more severe grades of EIPH typically expressed more IL-1 and INF-γ mRNA; therefore, collection of additional samples may have yielded additional significant results.

Although we did not report the origin or cell type involved for the present study, it has been reported elsewhere26 that intrapulmonary blood inoculation in horses initially causes a local neutrophilic infiltration, which is followed by macrophages and, to lesser degree, lymphocytes. Equine neutrophils can produce proinflammatory IL-1, IL-6, IL-8, and TNF-α mRNA instead of IL-4, IL-5, and INF-γ mRNA, which is mainly produced by lymphocytes.27 On the basis of this information, analysis of the results suggested that after EIPH-induced pulmonary neutrophilia, there may be an association between the observed systemic inflammation and neutrophils of pulmonary origin. However, because the origin of cytokines in horses with EIPH is unclear and may include alveolar macrophages, epithelium, endothelium, or stromal fibroblasts (and possibly other cells), this finding is speculative.

The mRNA expression of cytokines in a population of racing Thoroughbreds reported here may assist in determining the immunopathogenesis of EIPH. Gene linkage studies may prove useful in determining the susceptibility of horses to EIPH by allowing investigators to evaluate the balance of expression of proinflammatory and anti-inflammatory cytokines. Further research on therapeutic strategies, such as neutralizing antibodies, receptor antagonists, soluble receptors, and inhibitors of proteases, may be warranted.28 Use of these therapeutic strategies may interrupt the cascade of proinflammatory cytokines and reduce the prevalence and severity of EIPH.

Analysis of results of the study reported here indicate that increased production of IL-6 mRNA is associated with more severe forms of EIPH in horses that race at sea level and that altitude may affect proinflammatory (particularly TNF-α) and anti-inflammatory (particularly IL-6) cytokine expression. However, because
a cause-and-effect relationship was not established by this study, the pathophysiologic importance of these findings remains to be explained. Further research is required to evaluate more cytokines and protein expressions of cytokines and whether the inflammatory response observed in this study was attributable to pre-existing pulmonary inflammation or was a direct consequence of pulmonary bleeding.

b. Pentax Corp, Tokyo, Japan.
c. PAXgene RNA collection tubes, Qiagen, Valencia, Calif.
e. 7500 sequence detection system, Applied Biosystems, Foster City, Calif.
f. Fast Taq polymerase, Applied Biosystems, Biosystems, Foster City, Calif.
g. Applied Biosystems, Foster City, Calif.
h. Assay-by-Design kit, Applied Biosystems, Foster City, Calif.
i. EpMotion 5070, Eppendorf, Westbury, NY.
j. SYSTAT Software Inc, Chicago, Ill.

References


Appendix appears on the next page
### Appendix

Nucleotide sequences of equine-specific primers used in a real-time PCR assay.

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*Each primer was used at a concentration of 1.8 µM, and each probe was used at a concentration of 5µM.