

Alveolar Macrophage Function in Thoroughbreds after Strenuous Exercise

L. J. HUSTON, W. M. BAYLY, H. D. LIGGITT and N. S. MAGNUSON
Department of Veterinary Microbiology and Pathology and Veterinary
Clinical Medicine and Surgery, College of Veterinary Medicine, Washington
State University, Pullman, Washington, USA 99164-6610.

Summary

Horses subjected to strenuous exercise are prone to develop infectious pulmonary disease. Alveolar macrophages (AM) are an important initial component of pulmonary defense and their functional alteration may contribute to the development of pulmonary infection. The effects of a single bout of intense treadmill exercise on AM functions were determined on 5 unconditioned Thoroughbred horses. Bronchoalveolar lavage was performed once on each horse 30 minutes, or 1, 3 or 5 days after each exercise period. The sequence of lavage collection from each horse was randomized and the exercise test was repeated 4 times with at least 7 days between lavage and the next exercise. Recovered cells were quantitated and assayed for their ability to phagocytize antibody coated sheep erythrocytes (EAs). Serum cortisol and lavage fluid cortisol, lipid and protein concentrations were also evaluated. Numbers of cells recovered were significantly lower after exercise on day 3. There was a significant reduction in per cent EA-positive cells at 30 minutes and at 1 day after exercise, while the capacity of AM for EAs was significantly higher on day 3. Phagocyte viability measured after exercise was significantly lower at all times. Serum cortisol was significantly higher than control at 30 minutes and at 1 day, lavage fluid cortisol was elevated at 30 minutes and lavage fluid lipid/protein ratios were increased after exercise at all sample times. These results indicate that strenuous exercise has a deleterious effect on AM function. Such changes may help explain the increased prevalence of infectious respiratory disease in horses subjected to intense exercise.

Index terms: Bronchoalveolar lavage; phagocytosis; cortisol; pulmonary disease; bacterial killing.

Introduction

Depressed host defense is believed to be a common predisposing cause of infectious disease. Dysfunction of the immune system may occur for a number of reasons, one of which is stress. Stressful conditions which contribute to increased blood cortisol levels in horses include transport, strenuous exercise, parturition and psychologic stress such as pre-race excitement. Cortisol is a potent anti-inflammatory agent which acts

directly on cells of the immune system. Its effects may be viewed as beneficial in an acute inflammatory event, or as detrimental because of its ability to impair host defenses.

Of major concern to veterinarians and to the equine industry is the high incidence of respiratory tract disease which results from a combination of stress, exposure to influenza virus and invasion of bacterial opportunists such as *Streptococcus zooepidemicus*. Outbreaks are particularly common when large numbers of potentially stressed horses are housed in close proximity, as occurs at racetracks and large horse shows. The two most important individual clearance mechanisms of the respiratory tract are the mucociliary epithelial lining of the airways and the pulmonary alveolar macrophage (AM). The apparent susceptibility of Thoroughbred racehorses to respiratory tract disease following intense exercise led us to hypothesize that such activity may alter some component(s) of the defensive mechanisms within the equine respiratory tract. The effects of a single exercise bout on alveolar macrophage function indicate that strenuous exercise has a deleterious effect on this component of the equine mucosal defense system. This information improves our understanding of the pathophysiological effects of stress on lung defense mechanisms. This contribution to our knowledge may help initiate improved treatment or prophylactic measures against equine respiratory disease.

Materials and Methods

Animals. Horses used in this study were mature Thoroughbred horses from research herds at Washington State University College of Veterinary Medicine. Horses were housed in partially covered paddocks, fed alfalfa hay cubes and given free-choice water.

Exercise and lavage protocol. Five unconditioned horses were subjected to treadmill exercise for a duration of 9 min on a 10% grade. The horses were warmed up for 6 min at a walk (2 m/sec), trot (5 m/sec) and slow gallop (7 m/sec) and then exercised strenuously at 9 m/sec and 11 m/sec for 1.5 min at each speed. Bronchoalveolar lavage (BAL) was performed once on each horse after exercise at specified durations (30 min, 1, 3 or 5 days). Control data was gained from the results of lavages performed 7 days before exercise (-7 days), and 5 days after a lavage which was not associated with exercise. The horses were allowed a minimum of 7 days to recover from any effects of exercise and/or lavage before exercise was repeated. Each horse was tested and lavaged 4 times with the times between exercise and lavage being randomly sequenced for each horse. Each horse underwent all treatments and control lavages and served as its own control. Serum and BAL fluid samples from at least 3 time periods, including a control, were represented on each assay day. All exercise, blood sampling, and lavages were performed at the same time of day. Horses were mildly sedated with xylazine and butorphanol prior to lavage.

Macrophage collection. Alveolar macrophages were obtained by bronchoalveolar lavage using a polypropylene tube (Bev-a-line, Cole-Parmer Instruments Co. Chicago, IL) (0.8cm OD, 190cm long) connected directly to a 50 ml plastic syringe (Trigo *et al.*, 1984). This collection tube was passed through a shorter flexible PVC tube (Tygon #T6034-10, American Scientific Products, Redmond, WA) (0.9 cm ID, 1.4 cm OD, 80 cm long) which was inserted to midtrachea to reduce the chance of pushing nasopharyngeal bacteria into the lung. The 50 ml syringe was used to infuse and collect sterile 0.9% sodium chloride (Abbott Laboratories, North Chicago, IL) solution which was buffered with phosphate buffer, 0.05 M, pH 7.4. A total of 350 ml of phosphate

buffered solution (PBS) was introduced. Cells recovered after centrifugation were washed in RPMI 1640, counted with a hemacytometer and then resuspended in RPMI 1640 at a concentration of 1×10^7 cells/ml. Differential counts were made from cytocentrifuge preparations stained with Diff-Quik (American Scientific Products, McGraw Park, IL). Cell viability was determined by trypan blue dye exclusion. The quantity of lavage fluid recovered was measured and aliquots were filtered through 1.2 mm and 0.45 mm membranes (Millipore, Bedford, MA) and frozen at -20°C .

Phagocytic assay. Fc-receptor mediated phagocytosis was measured using the method of Liggitt *et al.* (1985). Alveolar macrophages were allowed 1 hour to adhere to glass coverslips, after which nonadherent AM were gently removed by washing with RPMI 1640. Sheep red blood cells (SRBC), sensitized with rabbit anti-SRBC antibody (Cordis Laboratories, Detroit, MI) (EA), were incubated with AM for 30 minutes. Nonphagocytized EA were lysed by direct exposure to water and coverslips mounted in trypan blue stain. The per cent of cells with 1 or more internalized EA and the number of EAs internalized per phagocytic cell were determined for 100 cells.

Cortisol assay. Serum and lavage fluid cortisol concentrations were measured using a ^{125}I radioimmunoassay kit (Gamma Coat, Travenol-Genentech Diagnostics, Cambridge, MA). Lavage fluid was lyophilized and resuspended in water to achieve a 25-fold concentration. Ten μl of serum or 30 μl of concentrated lavage fluid were used in the assay. Each sample was run in duplicate. A recovery of 97% of cortisol spiked lavage fluid was obtained.

Protein assay. Lavage fluid protein was measured by the Lowry assay modified by Edelson and Duncan (1981). Albumin protein (Sigma Chemical Co., St. Louis, MO) was used as the standard.

Lipid assay. Lipid determinations were made on 100 μl of 25 X concentrated lavage fluid. We modified the method of Toro and Ackerman (1975) by using 1 ml of sulfuric acid and 6 ml of phosphovanillin.

Statistical analysis. The results determined for each time period were compared using the t-test of paired observations. A value of $P < 0.05$ was considered statistically significant. The data are presented as mean \pm the standard error of the mean.

Results

The results from horses lavaged 5 days after an earlier lavage were not significantly different from results of control lavages on the same horses (-7 days) for any of the assays which were performed (data not shown). The concentration of recovered lavage cells was significantly reduced 3 days after exercise (Fig. 1). By 5 days, the numbers were increased to almost control levels. The total number of alveolar cells recovered in lavage fluid followed the same trend as the concentration of cells recovered. The amount of lavage fluid did not differ significantly at any time after exercise.

The proportion of cells that were alveolar macrophages, lymphocytes, PMN and eosinophils was not altered by exercise. We observed no morphological changes in AM after exercise although exercise did influence alveolar cell viability (Fig. 2). Reduced cell viability was evident at 30 minutes and was significantly less than in control levels 1, 3 and 5 days after exercise.

Cell function, as measured by evaluating phagocytosis of antibody coated sheep red blood cells (EA), was significantly impaired by exercise. The per cent of AM that

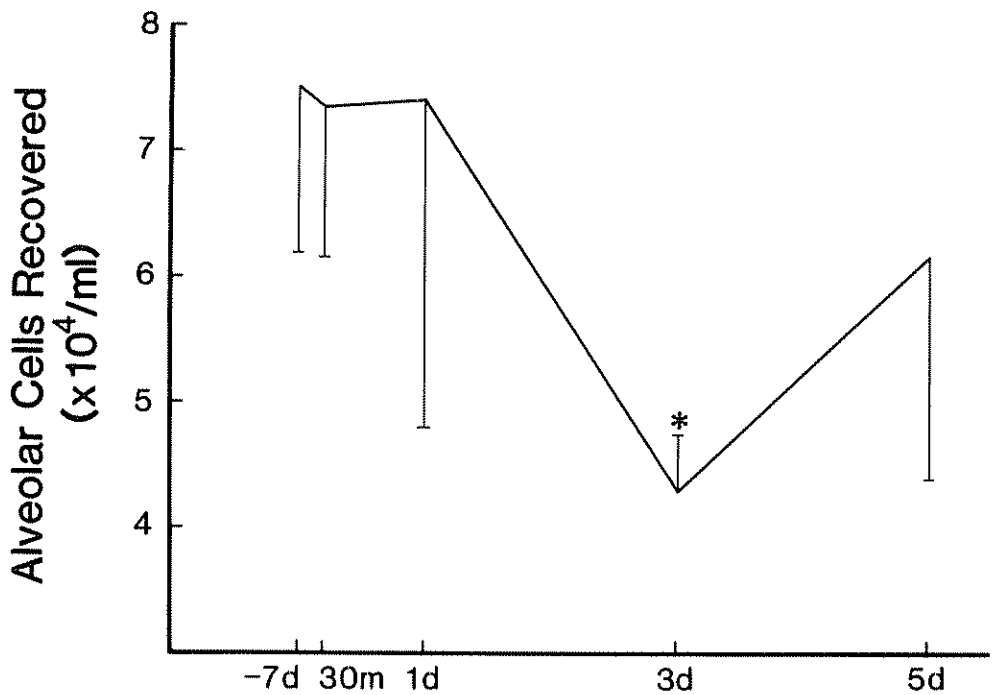


FIGURE 1. Alveolar cell recovery per ml of lavage fluid following treadmill exercise. * indicates a significant difference between this measurement and control (-7 day).

internalized one or more EA declined at 30 minutes after exercise and remained depressed after 1 day. At 3 and 5 days, the proportion of phagocytic positive cells returned to pre-exercise proportions (Fig. 3). The ability of AM to phagocytize a number of EA, called phagocytic capacity, is also shown in Fig. 3. Alveolar macrophages became progressively more efficient at phagocytosis, with significantly more EA per AM on day 3 compared to control levels.

Serum cortisol concentration was elevated in response to a single bout of intense exercise. It was significantly higher than pre-exercise levels at 30 minutes and at 1 day following exercise (Fig. 4). Cortisol measured in lavage fluid was also significantly elevated 30 minutes post exercise. When expressed as μg cortisol to g protein, there was a 3.5-fold increase over control levels at 30 minutes post-exercise (Fig. 4). No significant differences in lavage fluid protein were found, but cortisol to protein ratios differed significantly between horses (data not shown).

To estimate lavage fluid surfactant, we measured the lavage fluid lipid and also expressed it as a ratio of lipid to lavage fluid protein. Lavage fluid lipid was significantly higher than control at all sample times after exercise (Fig. 5).

Discussion

Stress, particularly that which occurs during transportation or hard physical exertion, is believed to play an important role in the pathogenesis of equine respiratory infections. Until now, there has been no objective evidence to substantiate this impression. Similar

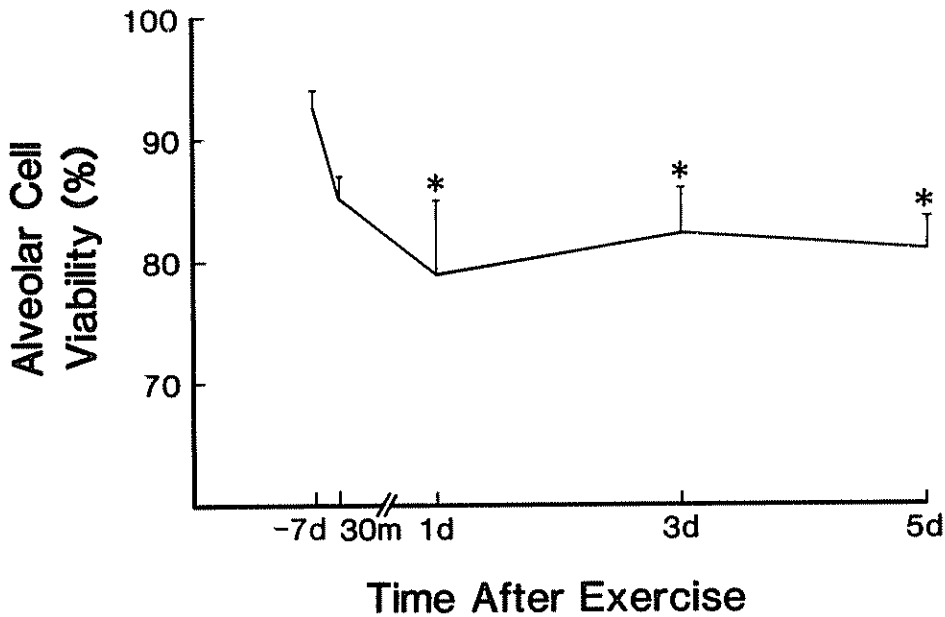


FIGURE 2. Viability of alveolar cells recovered via bronchoalveolar lavage before and after strenuous exercise. * indicates a significant difference between this measurement and control (-7 day).

suggestions have been made in cattle in which a respiratory disease syndrome, "shipping fever," results in significant economic loss (Jessen, 1976). The capacity of the pulmonary cellular and humoral immune defenses to defend against potentially infectious agents is fundamental to protection against respiratory disease. Several workers have shown that cattle infected with *Pasteurella* organisms only become clinically and pathologically affected if pulmonary clearance of the bacteria is impaired (Lopez *et al.*, 1976; Jericho and Langford, 1978). A normal, non-stressed animal challenged via the respiratory tract with *P. haemolytica* will rarely develop clinically significant pneumonia (Saunders and Berman, 1964; Jericho and Langford, 1978). The majority of infectious pneumonic conditions in horses involve opportunistic bacterial infections (Beech, 1979).

Therapeutic administration of a potent synthetic glucocorticoid such as dexamethasone (30 times more potent than cortisol) has been associated with adverse reactions in cattle (Roth and Kaeberle, 1982). In particular, treating cattle suffering from bronchopneumonia with dexamethasone, in the belief that it may have a beneficial effect by reducing the inflammatory response in the lungs, resulted in a poorer response to treatment, more relapses and increased mortality (Christie *et al.*, 1977). Westly and Kelley (1984), and Blecha and Minocha (1983), have shown that acute stress and elevation of blood cortisol concentration resulted in suppressed immunoresponsiveness in pigs and cattle.

Our results show serum cortisol levels in horses were elevated significantly 30 minutes and 1 day following intense exercise. These values are similar to values reported by Snow and McKenzie (1977) and Snow *et al.*, (1983), prior to and following racing

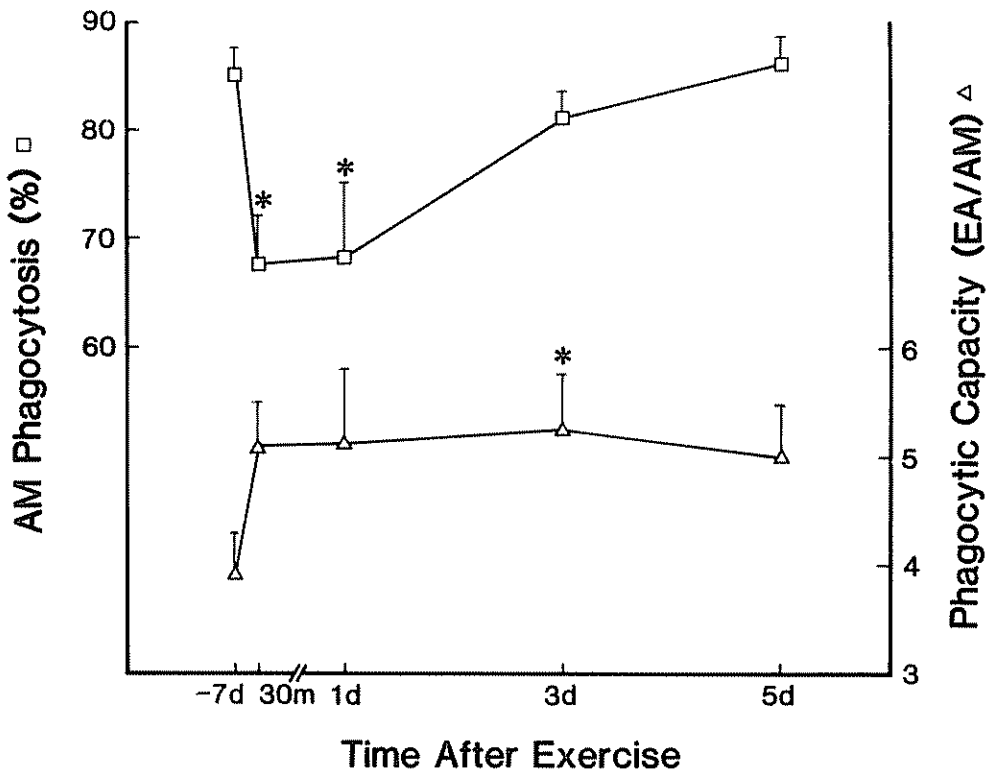


FIGURE 3. Fc-receptor mediated phagocytosis of antibody-coated sheep red blood cells before and after strenuous exercise. * indicates a significant difference between this measurement and control (-7 day).

or intense exercise. Intensity and duration of exercise influence the cortisol response (Thornton, 1985), as does the degree of fitness of the horse (Grosskope *et al.*, 1983). That cortisol remained high for 1 day after exercise may be evidence of continued stress. The lavage fluid cortisol concentrations were elevated at 30 minutes after exercise which indicates the relative ease of cortisol movement into the lung. Protein levels in lavage fluid obtained after exercise were not significantly different from pre-exercise levels, which suggests that vascular permeability was not altered by the exercise bout. No direct toxic effect of glucocorticoids on AM has been reported either *in vitro* or *in vivo*. The reduced viability of AM after exercise may therefore be due to exercise induced changes in the alveoli resulting in bystander lysis rather than due to direct action of cortisol.

Lung surfactant is essential in preventing the collapse of alveoli in normal animals. It consists of phospholipids, glycoproteins, and other components in smaller quantities. Synthesis of surfactant is stimulated by glucocorticosteroids and its secretion is stimulated by catecholamines (Rooney, 1985), both of which are elevated following intense exercise. Investigators have reported that concentrated lung lining material may augment AM function *in vitro* (Ando *et al.*, 1979; McNulty and Reason 1981). However,

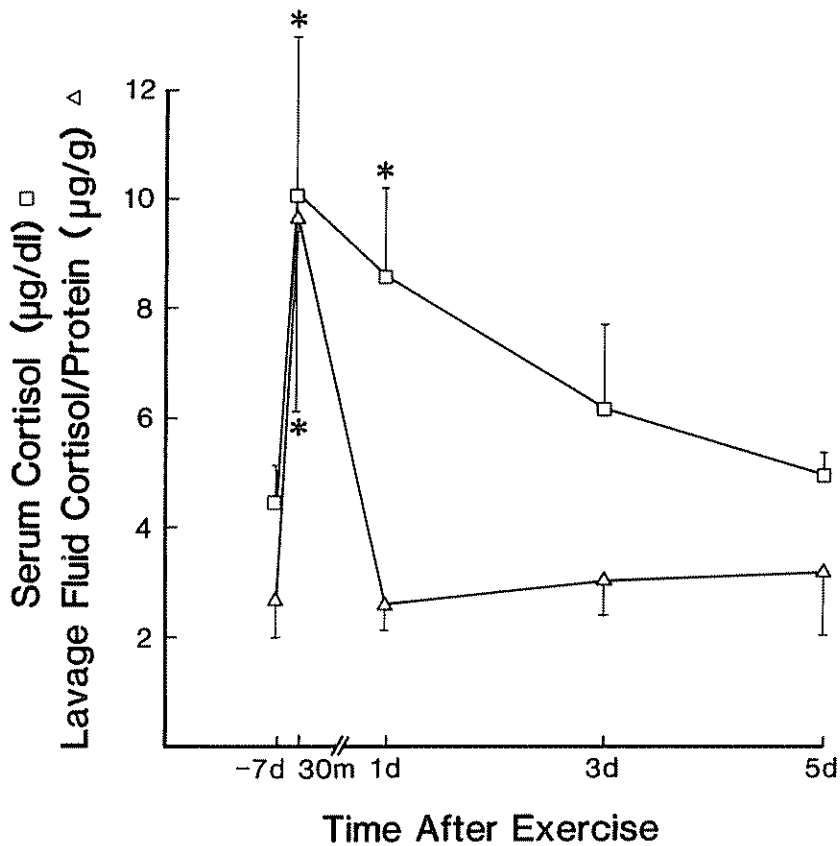


FIGURE 4. Serum cortisol concentrations and the ratio of lavage fluid cortisol to lavage fluid protein in horses before and after strenuous exercise. * indicates a significant difference between this measurement and control (-7 day).

one study found that it inhibited rather than enhanced phagocytosis of *P. haemolytica* (Liggitt, 1985). There is some evidence that infectious agents and transport result in the destruction of the alveolar type 2 pneumocytes which are responsible for the synthesis and secretion of surfactant (Liggitt *et al.*, 1983; Stinson *et al.*, 1983). The significance of increased surfactant in the lungs of exercise stressed horses is unknown. Lung alveolar lining material as a secretory product is not as well defined as are complement and interferon, but it may play an important role in the defense of the lung.

The ability of equine AM to phagocytize antibody coated SRBC after the stress of intense exercise has not been previously evaluated. Information on the influence of cortisol or synthetic glucocorticoids on AM of other species *in vitro* is incomplete and sometimes conflicting. Differences in results may be due to differences in technique, species, or the glucocorticoid evaluated (Gilka *et al.*, 1974; Pennington *et al.*, 1979). Stress-induced neurohormone release has also been implicated in immunosuppression (Blalock and Smith, 1985). Our results indicate that the proportion of AM which are able to perform phagocytic functions are impaired following a single bout of strenuous

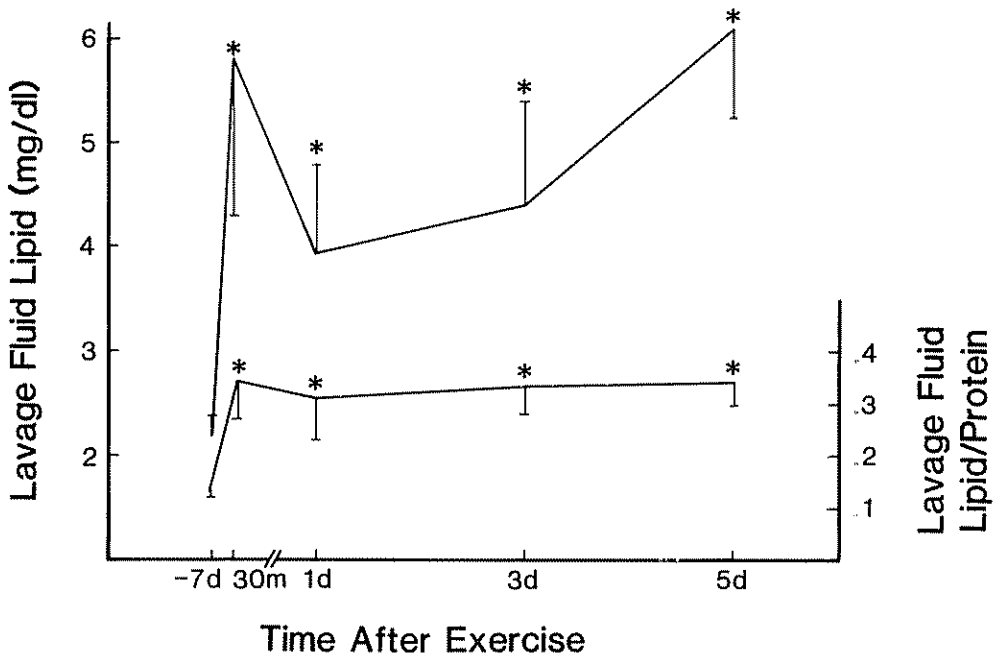


FIGURE 5. Lavage fluid surfactant measured as lipid in 5 horses following strenuous exercise. * indicates a significant difference between this measurement and control (-7 day).

exercise, although the mechanism responsible for this alteration is unknown. It is also difficult to judge the relative importance of various functional parameters. For instance, the increased Fc-receptor mediated phagocytic capacity of the AM that we observed, may have functionally compensated for the reduced proportion of phagocytic AM seen relatively soon after exercise. Further studies to be completed in our laboratory include quantitation of lavage fluid immunoglobulins and in vitro testing of the effects of cortisol and lavage fluid concentrates on AM function and viability.

The effects of repeated, daily bouts of intense exercise on stress hormones and alveolar macrophage function has yet to be determined. However, the results of this study suggest that a single bout of strenuous exercise has a deleterious effect on the function of a major component of pulmonary mucosal defenses, the AM. The changes reported here may be vital to the improved understanding of the pathophysiological events responsible for the increased prevalence of infectious respiratory disease in recently stressed horses.

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