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Research paper

Neutrophil function in healthy aged horses and horses with pituitary dysfunction

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ABSTRACT

Immunosuppression leading to opportunist bacterial infection is a well-recognized sequela of equine pituitary pars intermedia dysfunction (PPID). The mechanisms responsible for immune dysfunction in PPID however, are as of yet poorly characterized. Horses with PPID have high concentrations of hormones known to impact immune function including α-melanocyte stimulating hormone (α-MSH) and insulin. α-MSH and related melanocortins have been shown in rodents and people to impair neutrophil function by decreasing superoxide production (known as oxidative burst activity), migration and adhesion. The goal of this study was to determine if neutrophil function is impaired in horses with PPID and, if so, to determine if plasma α-MSH or insulin concentration correlated with the severity of neutrophil dysfunction. Specifically, neutrophil phagocytosis, oxidative burst activity, chemotaxis and adhesion were assessed. Results of this study indicate that horses with PPID have reduced neutrophil function, characterized by decreased oxidative burst activity and adhesion. In addition, chemotaxis was greater in healthy aged horses than in young horses or aged horses with PPID. Plasma insulin: α-MSH ratio, but not individual hormone concentration was correlated to neutrophil oxidative burst activity. In summary, neutrophil function is impaired in horses with PPID, likely due to altered hormone concentrations and may contribute to increased risk of opportunistic infections. Whether regulation of hormone concentration profiles in horses with PPID using therapeutic intervention improves neutrophil function and reduces infections needs to be explored.

1. Introduction

Immune dysfunction is a well-recognized sequela of equine pituitary pars intermedia dysfunction (PPID) that often manifests as opportunist bacterial infections (Schott, 2002; McFarlane, 2011, 2014). The mechanisms responsible for immune deficiency in PPID are unknown. It has previously been suggested that high serum or tissue cortisol concentrations may represent the underlying hormonal imbalance that leads to clinical signs including immune dysfunction and for years PPID was called equine hyperadrenocorticism or equine Cushings disease (Schott, 2002; McGowan and Neiger, 2003; Johnson et al., 2004). However evidence that cortisol is typically elevated in horses with PPID is lacking. In contrast, α-melanocyte stimulating hormone (α-MSH), a primary product of prohormone, proopiomelanocortin (POMC) processing in the pars intermedia, is reliably increased in PPID

Abbreviations: ACTH, adrenocorticotropin; α-MSH, alpha melanocyte stimulating hormone; HBSS, Hank's buffered saline solution; LTB4, leukotriene B4; PAF, platelet activating factor; POMC, proopiomelanocortin; PPID, pituitary pars intermedia dysfunction.

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(Wilson et al., 1982; Orth et al., 1982; Beech et al., 2009; McFarlane et al., 2004). α-MSH is known to have potent immunomodulatory effects in people and rodents (Catania and Lipton, 1993; Lipton and Catania, 1997; Brzoska et al., 2008). α-MSH and related melanocortins have been shown to impair neutrophil function by decreasing superoxide production (known as oxidative burst activity), migration and adhesion (Catania et al., 1996; Oktar et al., 2004; Manna et al., 2006; Capsoni et al., 2007; Bijuklic et al., 2007). α-MSH also blunts NF-κB activation thus arresting initiation of the inflammatory cytokine cascade (Manna and Aggarwal, 1998; Taylor, 2005). In light of the apparent predilection for PPID horses to develop bacterial infections, we hypothesized that neutrophil function may be impaired in horses with PPID due to excessive plasma α-MSH.

The goal of this study was to determine if neutrophil function is impaired in horses with PPID and, if so, to determine if plasma α-MSH concentration correlated with the severity of neutrophil dysfunction. Furthermore, because PPID is a disease that affects aged horses, a secondary objective was to determine if aging alone had a detrimental effect on neutrophil function in the horse. Specifically, neutrophil phagocytosis, oxidative burst activity, chemotaxis and adhesion were assessed.

2. Materials and methods

2.1. Animals for study

All procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Oklahoma State University. Sixty two horses were included. The assessment of neutrophil oxidative burst activity, phagocytosis and chemotaxis (Study 1) was performed in 13 horses with PPID and 24 healthy horses, of which 13 were adults (age range, 4–15 yrs), and 11 were aged (age > 20 yrs). All samples were collected from Aug 1 to Oct 31. Samples for neutrophil adhesion (Study 2) were collected from an additional 10 horses with PPID and 15 healthy horses. Samples were collected for adhesion studies Jun 1–Jul 31. Horses were diagnosed with PPID based on the presence of clinical signs and increased plasma ACTH concentration using seasonally specific reference ranges or necropsy confirmation of pars intermedia micro or macroadenoma(s). Necropsy confirmation of disease status was available in 12/13 PPID, 7/13 adults, and 11/11 aged horses in Study 1 and 9/10 PPID and 6/15 healthy horses in Study 2. All horses without a necropsy were monitored for a minimum of 2 additional years without change in clinical PPID status.

2.2. Collection of blood samples

All blood samples were obtained between 7:30 am and 11:00 am by venipuncture using vacuum tubes containing either heparin (neutrophil studies) or EDTA (hormone assays) (Becton Dickenson Vacutainer Systems, Rutherford, NJ, USA). Samples for neutrophil studies remained at room temperature and were processed within 1 h. EDTA blood samples were collected and placed on ice and processed within 1 h.

2.3. Radioimmunoassays: α-melanocyte stimulating hormone (α-MSH), cortisol, insulin and adrenocorticotropin (ACTH) analysis

Plasma ACTH and α-MSH concentrations were measured using commercial competitive, double antibody radioimmunoassays previously validated for use with equine plasma (MP Biomedical, Orangeburg, NY; Eurodiagnostica, Malmo, Sweden, respectively) (Cordero et al., 2012; McFarlane et al., 2004). Insulin and cortisol concentrations were determined using previously validated solid phase radioimmunoassays (Coat A Count, Siemens, Los Angeles, CA) (Banse et al., 2014; Cordero et al., 2012).

2.4. Neutrophil isolation

Heparinized venous blood samples were collected and neutrophils were isolated by density centrifugation through Ficoll (Histopaque 1077; Sigma, St Louis, MO). Briefly, heparinized blood samples were allowed to stand stationary for approximately 30 min at room temperature to allow the red blood cells to sediment. Subsequently, the upper leukocyte rich plasma layer was aspirated, layered over 20 ml of Ficoll in a 50 ml conical tube and centrifuged for 30 min at 400 g at room temperature. The mononuclear layer was discarded and the red blood cell, neutrophil layer was resuspended in 2 ml of distilled water to lyse the red blood cells. The neutrophils were washed twice, resuspended in HBSS and cell concentration determined using a hemocytometer. Concentration of neutrophils was adjusted to 2×10^6 cells/ml for Study 1 or 10^7 cells/ml for Study 2.

2.5. Phagocytosis and oxidative burst analysis

Standard flow cytometry methods were employed for assessment of oxidative burst, and phagocytosis. Simultaneous flow cytometric analysis of phagocytosis and oxidative burst activity was performed as previously described with slight modification (Flaminio et al., 2002; Holbrook et al., 2012). Neutrophils were resuspended in HBSS at a concentration of 2×10^6 cells/ml and stored on ice. Killed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem-Novabiochem Corp., La Jolla, CA, USA) were labeled with propidium iodide (PI). PI (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) was diluted to 50 μg/ml in 0.1 M Carbonate Buffer at 9.6pH. Subsequently, 1 ml of PI solution was mixed by vortexing with 50 μl of Pansorbin and incubated overnight at 4 °C. Next, the Pansorbin/PI solution was washed twice in an equal volume of cold HBSS followed by centrifugation in a microcentrifuge at 11,000 rpm for 1 min. The Pansorbin/PI was opsonized by incubation with 40% pooled equine serum at 37 °C for 30 min, followed by two additional wash steps in cold HBSS. The opsonized PI labeled killed *S. aureus* were resuspended in 1 ml cold HBSS. Dihydrorhodamine 123(DHR) (Molecular Probes Inc., Eugene, OR, USA) was dissolved in DMSO at a concentration of 29 mM and stored at −80 °C. Working solution of DHR (25 μg/ml) was prepared by diluting the cold HBSS, and kept in the dark until use. Simultaneous measurement of phagocytosis and oxidative burst