

Adherence as an *In Vitro* Assessment of Neutrophil Function in Alpacas

by
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Camelids are known for frequent occurrence of general ill-thrift. In other species, neutrophil dysfunction has been identified as a cause of ill-thrift, and linked to chronic fungal and bacterial infections. However, no reference values exist for neutrophil function testing in camelids, which inhibits accurate evaluation of neutrophil deficiency in this species. Neutrophil adhesion values were investigated in six healthy alpacas. Neutrophils were isolated from whole, EDTA-treated blood once per week for three weeks and suspended in RPMI 1560, supplemented with 1% heat-inactivated fetal bovine serum. Five hundred thousand cells were added to each well in a 96-well non-tissue culture treated plate and either untreated or stimulated with phorbol 12-myristate 13-acetate (PMA). Non-adherent cells were discarded, adhered cells were stained with crystal violet gram staining solution, and plates were washed with deionized water. Stain was solubilized with 1% SDS and percent adherence determined by absorbance at 570 nm. No significant difference was found in the percent neutrophil adherence between weeks ($p = 0.59$). Mean percent neutrophil adhesion was $12.4\% \pm 3.0\%$ ($9.4\% - 15.4\%$). Given that results from individual alpacas appear to be relatively consistent at different

time points, use of this adhesion assay may be a valuable tool in investigating neutrophil function in camelids.

Key Words: neutrophil, function, camelids, alpacas, adhesion

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Adherence as an *In Vitro* Assessment of Neutrophil Function in Alpacas

Introduction

Importance of Neutrophil Function Testing

Camelids are known for their mysterious immune systems and frequent occurrence of general ill-thrift (Davis et al., 2000). While recent research has provided insight into several of the underlying mechanisms of ill-thrift, many occurrences are yet to be explained. In some cases, underlying immunodeficiency has been identified (Hutchison et al., 1995; Davis et al., 2000). Investigations into immunocompromised camelids have focused on lymphocyte and lymphocyte deficiencies, specifically juvenile llama immunodeficiency syndrome (Hutchison et al., 1995; Davis et al., 2000). The roles of other immune cell types have not been investigated. In other species, dysfunctions of neutrophils have been linked to ill-thrift, as seen in bovine leukocyte adhesion deficiency in cattle, and neutropenia, such as cyclic neutropenia in grey collies, results in similar clinical presentation to neutrophil deficiency (DiGiacomo et al., 1983; Nagahata et al., 1993). The neutrophil is a short-lived white blood cell that plays key roles in the inflammation response (Parkin and Cohen, 2001; Summers et al., 2010). Deficiencies in neutrophil function often increase susceptibility to fungal and bacterial infections, which in turn leads to the ill-thrift (Nagahata et al., 1993; Summers et al., 2010)

The overall function of neutrophils can be broken into several distinct components, some of which can be tested *in vitro*.

Little research has been done to establish a connection between poor-performance and neutrophil function, which may be in part because no standard reference ranges for

the results of neutrophil function tests appear to exist in camelids. It should be noted that quantitative assessment methods of neutrophil function in the other livestock species are well established (DiGiacomo et al., 1983; Cebra et al., 2003; Hall et al., 2006; Raboisson et al., 2014).

The absence of standard values for innate immune function in camelids provides numerous obstacles to further research and development of diagnostics in camelids in both academic and clinical settings. Without standards for neutrophil function, the scientific community lacks the ability to diagnose neutrophil mechanism deficiencies or further study the effects of vitamins, minerals, diet, or other disease processes on the action of neutrophils in the body (Cebra et al., 2003; Summers et al., 2010; Raboisson et al., 2014). Establishing a standard range for neutrophil function is pertinent for the continued advance of camelid immune response in research and in medicine.

Immune System Overview

The immune system is a complex network and is comprised of two function subsystems: the innate and adaptive immune systems (Parkin and Cohen, 2001). The innate immune system is comprised of cellular elements, such as monocytes, macrophages, and neutrophils, as well as the complement system, and functions in cell recruitment, cytokine release, bacterial-killing, antigen-presentation, and activation of the complement cascade (Parkin and Cohen, 2001). The innate immune system is the body's first defense against infection and is quick to respond but lacks specificity (Parkin and Cohen, 2001). However, while the innate immune system may be somewhat primitive, it

is vital for animal survival and processes within the innate immune system are highly conserved throughout the animal kingdom (Parkin and Cohen, 2001).

The adaptive immune system is seen in higher-order animals and functions in recognition of specific pathogens and long-term immunity (Parkin and Cohen, 2001). T-lymphocytes and B-lymphocytes are pertinent to the adaptive immune response, as these cellular types possess the ability to create antigen-specific receptors (Parkin and Cohen, 2001).

The complement system is a network of zymogens, proteins, receptors, and regulators that are present within blood, fluids, and tissues and is fundamental to the inflammatory and immune response (Kościelska-Kasprzak et al., 2014). The complement system is activated in response to foreign pathogen identification as well as in response to apoptotic and ischemic cells and tissues (Kościelska-Kasprzak et al., 2014). Activation of the complement system functions as an enzyme cascade as complement zymogens are cleaved at the site of infection and become active (Kościelska-Kasprzak et al., 2014). The cascade is quickly amplified to result in a potent complement response (Parkin and Cohen, 2001; Kościelska-Kasprzak et al., 2014). The complement system can be activated via three pathways: the classical, alternative, and lectin pathways and functions via three major mechanisms (Parkin and Cohen, 2001; Kościelska-Kasprzak et al., 2014). The first mechanism is opsonization, which is the generation of activated complement proteins called opsonins that covalently bind to pathogens to target them for destruction by phagocytosis (Kościelska-Kasprzak et al., 2014). The second mechanism is the release of chemoattractant to recruit phagocytes, such as neutrophils, to the site of infection (Kościelska-Kasprzak et al., 2014). The third mechanism is the creation of complement

components that insert themselves into bacterial membranes to cause shifts in osmolarity and cause destruction of the bacterium via lysis (Kościelska-Kasprzak et al., 2014).

Neutrophil Function

Neutrophils, basophils, and eosinophils are classified as granulocytes due to the characteristics appearance of their granules, and are also known as polymorphonuclear leukocytes due to the characterization and appearance of the lobes of the nucleus (Haynes and Fletcher, 1990; Clark and Nauseef, 2001). Neutrophils constitute 60–80% of the granulocyte population in most animals (Davis et al., 2000) and are short-lived with a circulating half-life of 6–8 hours (Summers et al., 2010). Neutrophils are produced via hematopoiesis in the bone marrow and due to their brief circulating life, they are produced at rates of 5×10^{10} to 10×10^{10} cells per day, which results in a rapid turnover of cell population (Haynes and Fletcher, 1990; Summers et al., 2010). Neutrophils are present in high concentrations in the peripheral vascular system of the body and migrate into tissues once activated (Yakuwa et al., 1989; Davis et al., 2000).

Adhesion proteins expressed on the vascular endothelium aid in the migration of neutrophils through the vascular wall and into tissue expressing chemoattractant. Adhesion is thought to be the first step in extravascular migration of peripheral blood neutrophils (Yakuwa et al., 1989; Haynes and Fletcher, 1990). While in circulation, neutrophils appear smooth and spherical, but become amoeboid once they adhere to the vascular endothelium (Friedl et al., 2001; Summers et al., 2010). This change in cellular shape allows for chemotaxis towards chemoattractants (Friedl et al., 2001). Neutrophils possess several mechanisms for immune function, including phagocytosis of opsonized

bacteria, superoxide formation and subsequent oxidative burst, extrusion of neutrophil extracellular traps (NETs), and secretion of cytokines such as interleukin-8 (Haynes and Fletcher, 1990; Altstaedt et al., 1996; Amulic and Hayes, 2011). While adhesion is not directly inhibiting or killing invading pathogens, adhesion is necessary for the neutrophils to access the site of infection as well as adhere to bacteria for phagocytosis. Adhesion is therefore a parameter reflective of the activated state of neutrophils and *in vitro* neutrophil function (Yakuwa et al., 1989; Haynes and Fletcher, 1990).

The objective of this study was to establish baseline information for neutrophil adhesion in healthy camelids such that subsequent studies can further investigate the effects of stressors and micronutrients on neutrophil function.

In Vitro Neutrophil Function Tests

The moment a needle pierces a vein, the function of neutrophils have been effectively altered (Haynes and Fletcher, 1990). Additionally, blood collected through venipuncture contains non-adhered neutrophils, which may be functionally different from those attached to the vessel wall. For these reasons, *in vitro* neutrophil function tests are not necessarily an accurate depiction of neutrophil function *in vivo*, but are a reliable reflection of neutrophil state (Haynes and Fletcher, 1990).

Sterility when performing neutrophil function tests, while recommended, is not required due to the fact that neutrophils are terminally differentiated, and therefore have short life spans and cannot be propagated via cell culture (Haynes and Fletcher, 1990). Neutrophil function tests must be performed quickly after blood draw (preferably within 3 hours) as cell viability will continuously decrease *in vitro* (Haynes and Fletcher, 1990).

This allows for flexibility in the lab as long as there is focus on preventing contamination of samples (Haynes and Fletcher, 1990).

Several agents are commonly utilized *in vitro* to induce activated states in neutrophils, including tumor necrosis factor- α (TNF- α), N-Formylmethionine leucyl-phenylalanine (fMLP), and phorbol 12-myristate 13-acetate (PMA) (Yakuwa et al., 1989). PMA has been shown to stimulate neutrophils *in vitro* (Lo et al., 1988; Yakuwa et al., 1989) and in brief, works through the activation of protein kinase C within target cells (Wolfson et al., 1985; Benna et al., 1994; Raboisson et al., 2014). This same pathway is responsible for the stimulation of respiratory burst (Raboisson et al., 2014).

It is common to supplement cellular medium with heat-inactivated fetal bovine serum to provide necessary proteins for cellular viability (Haynes and Fletcher, 1990), and the process of heat-inactivation is necessary to destroy complement proteins that would interfere with the *in vitro* function of cells (Yakuwa et al., 1989; Haynes and Fletcher, 1990).

Materials and Methods

Animals and Experimental Design

Six alpacas from the Oregon State University Research Herd were selected for this study. All animal protocols were approved by Oregon State University's International Animal Care and Use Committee. Animals were housed in pairs at the Oregon State University Lois Bates Acheson Veterinary Teaching Hospital and were provided hay and water ad libitum. Blood was collected sterilely via jugular venipuncture into EDTA vacutainers every other week for a total of three collections per animal over the duration of the study. An additional 2 ml were drawn per animal into a 2 ml EDTA vacutainer and was submitted to the Oregon State Veterinary Diagnostic Laboratory for a CBC.

Neutrophil Isolation

Isolation procedures performed were a modification of two previous methods (Haynes and Fletcher, 1990; Clark and Nauseef, 2001). In brief, whole blood (EDTA) was centrifuged at $450 \times g$ for 45 min at room temperature. The plasma layer was removed and the buffy coats plus a small volume of red blood cells immediately underneath were transferred into a 50 ml centrifuge tube while supernatant and remaining erythrocytes were discarded. Buffy coats were washed once with a buffered, hypertonic ammonia chloride erythrocyte lysis solution (0.15 M NH_4Cl (ammonium chloride), 0.001 M NaHCO_3 (sodium bicarbonate), and 0.001 M ethylenediaminetetraacetic acid) in a volume double that of the collected buffy coat. Blood was mixed with lysis solution by

rocking and centrifuged $400 \times g$ for 15 min at room temperature. The supernatant was discarded and the same volume of fresh buffered lysis solution added. Tubes were rocked until complete lysis occurred (approximately 5–10 minutes) and then centrifuged at $400 \times g$ for 15 min at room temperature.

Lysis supernatant was discarded and the cell pellet suspended in approximately 1 ml of Ca- and Mg-free Hanks' Balanced Salt Solution without phenol red (Lonza, Portsmouth, NH), supplemented with 1% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA; HBSS+HIFBS) and transferred to a 1.5 ml Eppendorf tube. Cells were centrifuged at $300 \times g$ for 5 min at room temperature to wash the cells. This wash step was repeated two more times to remove residual lysis buffer. After the final wash, the supernatant was removed, cell pellet re-suspended in 200 μ l of HBSS+HIFBS and layered on top of 1 ml Lymphocyte Separation Medium (Corning, Manassas, VA; LSM) and centrifuged in a swinging bucket centrifuge at $400 \times g$ for 20 min at room temperature. The upper layer containing lymphocytes and residual LSM was removed and the neutrophil-rich pellet was washed three times in the manner described above and re-suspended in 1 ml of HBSS+HIFBS. The cell solution was counted via hemocytometer and cells were re-suspended in RPMI 1560 (Life Technologies, Eugene, OR), supplemented with 1% heat-inactivated fetal bovine serum (RPMI+HIFBS) at 5×10^6 cells/ml for use in the adhesion assay.

Adhesion Assay

The adhesion assay was a modification of a previously published method (Yakuwa et al., 1989). Five hundred thousand neutrophils in 100 μ l were added to

individual wells in a sterile, non-tissue culture treated 96-well plate (Corning Inc., Durham, NC). Half the wells were unstimulated (control) and the other half were stimulated with 1 ng/well of phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO; PMA). The plate was centrifuged at $50 \times g$ for 2 min and incubated at 37° C in a 95% CO₂ incubator for 60 min. After incubation, the plate was vortexed intermittently for 40 repetitions to remove non-adherent cells and supernatant was immediately discarded. Two hundred microliters of crystal violet solution (Hardy Diagnostics, Santa Maria, CA) was then added to each well and allowed to incubate at room temperature for 30 min. The staining solution was discarded and the plate rinsed with deionized water and allowed to air dry. The stain was solubilized by adding 100 μ l/well of 1% SDS (Amresco, Solon, OH) and quantitative evaluation of neutrophil adherence determined by absorbance measured at 570 nm (Multiskan Go, Thermo Fisher Scientific, Waltham, MA).

Statistical Analysis

An Anderson-Darling test was performed to test for normality. Percent adherence data was considered normally distributed with $p = 0.02$. Percent adherence was found for Week 2 and Week 3 and a paired t-test with $\alpha = 0.05$.

Results

Data from all six animals during the first week of blood draws were removed from consideration due to weak lysis buffer, clotting of samples, and over-handling of cells resulting in loss of cell viability when plated. Adherence data from Alpaca 2 (“Beau”) was excluded from the statistics due to the presence of a large fungal ulcer on his right hip. Beau’s CBCs revealed neutrophilia (Week 2 count of 25,193 and Week 3 of 30,108, reference range: 4,711 – 14,686), high neutrophil percent, and high lymphocyte percent on every day that a blood draw was performed. CBC data from Alpaca 3 (“Limerick”) during Week 2 revealed high neutrophil percent of 71% (reference 16% – 59%). During Week 3, Limerick’s CBC revealed neutrophil percent of 59%, which is the high boundary of normal for this species. All other animals enrolled in the study had normal CBCs results.

Cell morphologies were noted to be rough for Beau in Weeks 2 and 3 and for Limerick in week 2. Cell morphologies for all other animals in Weeks 2 and 3 were fair to excellent for unstained cells at counting.

Overall mean adherence percent was $12.4\% \pm 3.0\%$ ($9.4\% - 15.4\%$) with a range of 9.8% to 20.0% (see Table 1). Paired t-test yielded no significant difference between the two weeks ($p = 0.59$).

Table 1. Summary of Percent Neutrophil Adherence

Animal	Week 2	Week 3
Alpaca 1 – Andy	12.4	10.0
Alpaca 2 – Beau†	—	—
Alpaca 3 – Limerick	20.0	10.3
Alpaca 4 – Chloe	9.8	11.7
Alpaca 5 – Elm	11.9	13.1
Alpaca 6 – Ophelia	11.2	13.8
Mean ± SD	13.0 ± 4.0	11.8 ± 1.7
Total Mean ± SD	12.4 ± 3.0	

† Beau's Week 2 and Week 3 percent adherence values were 21.777% and 20.798%, respectfully. Due to Beau's abnormal clinical presentation, his percent adherence values were not considered when performing statistical analysis.

Discussion

In this study, no significant difference was found in the percent adherence of neutrophils between the data from Week 2 and Week 3 ($p = 0.59$). Week 1 showed low percent adhesion values, most likely due to excessive exposure of neutrophils to lysing conditions due to sample clotting and weak lysis buffer. This was reflected in cell morphologies, which were noted to be rough during the first week, and again likely due to the over-handling of cells due to weak lysis buffer. The findings suggest that *in vitro* neutrophil adhesion testing yields reasonably consistent results in specific alpacas.

Due to the fungal skin ulcer, Beau received surgical intervention during the course of the study at Oregon State University's Lois Bates Acheson Veterinary Teaching Hospital. Beau's percent adherences for Weeks 2 and 3 were 21.8% and 20.8%, respectfully, considerably higher than the mean results for the group (13.2% and 11.8%, respectfully). Beau's CBCs reflected his immune state with noted neutrophilia, overall high WBC count, and low lymphocyte percent. These values are also consistent with a stress leukogram. These results suggest that *in vitro* adhesion testing in alpacas with active inflammatory lesions may yield higher percent adherence than healthy alpacas, but more data are needed to conform this.

Limerick's CBC revealed high neutrophil percent during Week 2, but the neutrophil percent had dropped back within reference range by the Week 3 venipuncture. Week 2 data for Limerick show a higher percent adhered, which is reflective of his CBC during that week. These abnormalities were minor.

The percent adhesion of $12.4\% \pm 3.0\%$ is low compared to the reference values for other species (Cebra et al., 2003; Hall et al., 2006). While it is possible that camelid

neutrophils yield lower *in vitro* adherence than other species, there are other possibilities to account for the discrepancies. These neutrophil isolation and adhesion assay protocols may result in decreased cell viability. It is common knowledge that camelid erythrocytes pose more challenges for successful lysing, and this results in increased exposure of neutrophils to lysis solution compared to other species whose erythrocytes may lyse sooner. This possibility of low adherence values due to laboratory protocols could be further investigated by performing adhesion assays on multiple species concurrently with identical protocols.

Another consideration of poor neutrophil function may be the health of the animals in the study. While all animals were considered normal (except for Beau), the Oregon State University Camelid Research Herd mostly consists of older animals. Age may be a factor in neutrophil function. These animals are also utilized routinely for camelid research and may present an abnormal stress response when undergoing jugular venipuncture. Other subclinical health issues also could not be excluded.

There is a growing interest in the impact that nutritional factors, such as dietary nitrogen, copper, selenium, and vitamin D, may have on neutrophil function (Arthington et al., 1995; Binder et al., 1999; Cebra et al., 2003; Weiss and Hogan, 2005; Raboisson et al., 2014). It is important to note that the Oregon State University Teaching herd does not receive selenium supplementation. Selenium supplementation plays an important role in the oxidative burst, and blood selenium levels may influence that aspect of neutrophil function (Hogan et al., 1990; Cebra et al., 2003; Weiss and Hogan, 2005). Other function may be affected as well, including chemotaxis and adherence (Binder et al., 1999; Cebra et al., 2003). Measuring blood selenium concentration in the Oregon State University

herd may provide some insight into why adherence in these alpacas was below that seen in other livestock species.

Although these camelids had low *in vitro* neutrophil adherence compared to other species, they were outwardly healthy and had no clinical pathologies indicative of disease. Whether that is the result of the technique, a specific influence on this group of alpacas, or an inherent property of camelid neutrophils will require further investigation. Given that results from individual alpacas appear to be relatively consistent at different time points, use of this adhesion test may be a valuable tool in further investigation of this question.

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