The Effect of

CO$_2$ and HEPES

On Bovine Neutrophil

Migratory Function

by

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ABSTRACT

The objective of this study was to investigate the effects of incubation with CO_2 and HEPES on bovine neutrophil chemotaxis assays. On each sampling day, blood was collected from four lactating cows (4-163 days in milk; n=12 cows total). Neutrophils were isolated and adjusted to 2×10^6 cells/mL in Hank's Buffered Salt Solution containing 5% fetal bovine serum. For the chemotaxis assay a 48 well chamber was used (Neuro Probe Inc., Gaithersburg, MD). The bottom wells of the chambers contained 28 µl of media (with or without HEPES) supplemented with either 100 ng/mL of interleukin-8 (IL-8) or 50 ng/mL of complement component 5a (C5a). A five µm polycarbonate membrane (Neuro Probe Inc., Gaithersburg, MD) was used. The top wells of the chamber were then filled with 50 µl of PMN suspension. The chambers were incubated for 60 minutes at 37°C either with or without 5% CO_2. Neutrophil migration was determined by adherence to the membrane and concentration in the bottom wells of the chemotaxis chamber. Chemotaxis toward IL-8 and C5a was higher than toward controls for all measures. Chemotaxis toward IL-8 was not different from chemotaxis toward C5a. When chemotaxis was assessed by adherence to the membrane, the presence of CO_2 during incubation decreased neutrophil (PMN) migration, but concentration of PMN in the bottom wells of the chamber increased in the presence of CO_2. Inclusion of HEPES in the assay media increased concentration of PMN in the bottom wells but did not affect adherence. There was a t. An interaction was found between HEPES and chemoattractant on Con of migrated PMN (P=0.002). When HEPES was present there was an increase in PMN

migration to negative control, C5a, and positive control wells compared to when HEPES was not present. However, this HEPES-induced increase was significantly higher for migration of PMN to IL-8.

There was an interaction between HEPES and CO_2 on Ad of migrated PMN (P=0.0001). When CO_2 was present HEPES increased Ad of PMN. However when CO_2 was not present there was no effect of HEPES on Ad of PMN. In conclusion when PMN were incubated with CO_2 there was a decrease in function observed by Adhesion measurements and concentration measurements, however more work is need to further understand this relationship.

Chapter 1

LITURATURE REVIEW

1.1 The Immune System- Innate and Adaptive Immunity

The main purpose of the immune system is to identify and destroy invading

pathogens (Ingvartsen et al.,2012). The immune system is divided into two branches,

the innate immune response, and specific or acquired immune response. Both branches

help fight and protect against pathogens. While these two immune systems contain

different types of cells they often work together to create a functioning and successful

immune response (Chaplin., 2010). The transition period time is usually associated

with a decrease in immune function for both innate and adaptive systems and an

increase in susceptibility to metabolic disorders and chronic infections

The innate response is quick, nonspecific, and can be activated by a number of

stimuli (Sordillo et al., 1997). This system includes a large number of cell types and

physical barriers (Sordillo et al., 1997; Parkin et al., 2001; Chaplin., 2010; Ingvartsen

et al., 2012). The innate immune response is the most important defense during the

first stage of infection and it is also the most important defense system in the bovine

mammary gland (Stevens et al., 2011). The innate immune system includes

polymorphonuclear leukocytes or neutrophils (PMN), monocytes, macrophages,

cytokines and acute phase proteins. Their job is to provide immediate protection to the

host (Sordillo et al., 1997; Parkin et al., 2001; Ingvartsen et al., 2012). Other aspects of

the innate immune system include the anatomical structural defenses of the body.

These include the tight junctions of the epithelial cells, the mucosal layer and cilia on

epithelial cells (Chaplin., 2010). In the mammary gland bacteria first enter through the teat canal where keratin can be found. This keratin acts as a barrier for trapping bacteria and also contains antimicrobial agents (Sordillo et al., 1997). This is the first line of anatomical defense and is followed by a cellular defense system. The innate response also processes a number of small active proteins including complement proteins and defensins. Toll like receptors (**TLR**) are another important part of the innate immune response to help identify molecules from the invading pathogen and signal the release of cytokines. Most of the anatomical defense systems of the innate immune system are always active, however the cellular processes are only activated when they interact with pathogenic molecules (Chaplin., 2010).

The innate immune cells have a variety of functions to help destroy foreign pathogens. The first main function of the two main innate immune cells, PMN and macrophages, is to identify the pathogen (Ingvartsen et al., 2012). The next step is to produce cytokines such as interleukin (**IL**)-1β or tumor necrosis factor- alpha (**TNF-α**). These cytokines have functions including initiating both the innate and adaptive immune system and recruiting PMN to sites of infection. The third response of these cells is to phagocytose and kill the invading pathogen. The last step is the antigen presenting step through the use of the major histocompatibility complex (**MHC**). This step is how the innate and the adaptive immune system communicate (Ingvartsen et al., 2012).

The second half of the immune system is the adaptive immune system. This response is specific and consists mainly of B and T lymphocytes (Parkin et al., 2001). Both T and B lymphocytes can undergo clonal expansion (Chaplin, 2010). The response also occurs after the innate response. This response can sometimes take

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weeks to develop and requires memory cells, so that when the same pathogen re-enters the body is able to respond faster to the attack.

1.1.1 Inflammation

Acute inflammation occurs when there is an accumulation of neutrophils in the tissue (Mulder et al., 1993). The first step of the inflammatory process is when endotoxin or lipopolysaccharide (LPS) sheds off gram-negative bacteria and binds to TLR4 on macrophages to initiate the inflammatory response (Revelo et al., 2012). There are other TLRs that bind to gram-positive bacteria when LPS is not present. Neutrophils migrate toward and enter the site of infection by chemotaxis and diapedesis (Stevens et al., 2011). If this step is triggered when a pathogen is not present or if the response is out of control it will lead to inflammatory diseases like vasculitis, mastitis, laminitis, and other systemic inflammatory problems (Parkin et al., 2001; Stevens et al., 2011). Endotoxins stimulate macrophages and monocytes to secrete cytokines that cause PMN migration to the site of infection. When leukocytes respond quickly the animal gets better faster. However, prolonged diapedesis of leukocytes can lead to inflammatory disorders (Sordillo et al.,1997)

1.2 Neutrophils

The PMN has a polymorphic segmented nucleus, cytoplasmic granules used to kill pathogens, large stores of glycogen used for energy, and membrane receptors. There are many different types of receptors found on the PMN membrane; types of receptors include chemoattractant receptors, adhesion receptors, and complement protein and immunoglobulin receptors (Paape et al., 2003). Neutrophils are usually the first cell type to respond in the inflammatory response. They usually migrate quickly

toward chemoattractants, even when the chemoattractant gradient is low (Lokuta et al., 2007). Neutrophils kill invading pathogens by phagocytosis and intercellular killing (Ryman et al., 2013). Neutrophils can also create neutrophil extracellular traps, which trap pathogens in one place and stop them from spreading throughout the tissues (Revelo et al., 2012).

1.2.1 Cytokines

Cytokines are small molecular weight proteins produced by cells that control and regulate both the innate and specific immune system (Sordillo et al., 1997; Parkins et al., 2001). Cytokines aid in cell communication by binding to specific cell surface binding receptors. They can cause a cell to do many functions including activation, division, apoptosis, or movement (Parkins et al., 2001). The major groups of cytokines involved with PMN function are IL, colony stimulating factors (**CSF**), TNF, and interferons (**IFN**; Sordillo et al., 1997). Interleukins is a name given to a cytokine group that is produced by a leukocyte and affects other white blood cells, including PMN (Parkins et al., 2001). When a pathogen enters the body endotoxins or other substances are detected by TLRs, which cause leukocytes such as macrophages to release cytokines such as TNF-α and ILs (Parkin et al., 2001; Kim et al., 2005). Cytokines have been shown to play an important role in PMN apoptosis. During inflammation several cytokines are upregulated, including IL-1α, IL-2, IL-6, IL-8, IL-15, IFN-γ, CSF2, and CSF3. These cytokines all prolong PMN life span in human PMN (Paape et al., 2003). Tumor necrosis factor-α is also upregulated during inflammation; however it causes apoptosis. Cytokines also play a major role in inducing chemotaxis in PMN. Some major cytokines and cytokine-like molecules that can act as chemoattractants are C5a, C3a, LPS, IL-1, IL-2, and IL-8 (Paape et al.,

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2003). These molecules initiate their response by binding to specific receptors on the PMN membrane. Interleukin-8 can act as a chemoattractant but also has an important role in controlling the release of alkaline phosphatase from secondary granules and the production of reactive oxygen species (**ROS**). It also plays an important role as a chemoattractant during mastitis (Paape et al.,2003). Cytokines are released during the first stages of intra-mammary infection. The main cytokine involved in mastitis is IL-8 and is responsible for attracting PMN and causing the release of lysosomal enzymes (Ryman et al., 2013).

The complement system is made up of glycoproteins and functions in both the innate and adaptive immune response (Parkin et al., 2001). These proteins are activated in a cascade-like pathway, where the activation of one protein leads to the activation of the next. The three pathways that lead to the activation of the complement pathway are the classical pathway, antigen-antibody reaction pathway, and alternative pathway (Parkin et al., 2001). All three pathways lead to the activation of C3. The main purpose of the complement pathway is to form a membrane attack complex in the pathogen's cell membrane to cause the cell to burst by osmotic stress. C5 is a main protein involved in this complex and when cleaved by C5 convertase forms the chemoattractant C5a (Parkin et al., 2001). A chemokine is a specific cytokine whose main function is to stimulate leucocyte migration. Chemokines function as chemoattractants (Parkin et al., 2001).

1.2.2   Chemotaxis and Diapedesis

Migration of PMN from the blood into the tissues during periods of inflammation is a multi-stepped process that involves PMN rolling, activation, adhesion, and migration through the vascular endothelium (Huang et al., 2006).

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Adhesion is the process of the PMN adhering to endothelium to begin to move to the site of infection. Adhesion of PMN to receptors on the endothelial cell is mediated by cytokines that are used as cell messengers (Parkin et al., 2001). Adhesion molecules are important for cell communication, migration, and phagocytosis. The main adhesion molecules are intercellular adhesion molecules (**ICAM**), integrins, selectins, and cadherins and are expressed on both PMN and endothelial cells (Parkin et al., 2001; Huang et al., 2006). When a pathogen first enters the body macrophages and mast cells are activated and release TNF-α and IL-1 in response to pathogen associated molecular patterns including endotoxin (Parkin et al., 2001). This causes the vascular endothelium cell to express E-selectin which the PMN L-selectin can now bind to (Mulder et al., 1993; Parkin et al., 2001). The movement of PMN along these molecules is known as rolling (Parkin et al., 2001). After binding to E-selectin there is a rapid down-regulation of the L-selectins and an up regulation of integrins CD11 and CD18 found on the surface of PMNs. These new adhesion molecules promote binding to ICAM-1 (Mulder et al., 1993; Parkin et al., 2001). Adhesion molecules including PECAM-1, JAM-A, CD99, ICAM-1 and ICAM-2 are all important for PMN transmigration or diapedesis (Huang et al., 2006). ICAM2 is also shown to be important in leukocyte migration, activation, and angiogenesis (Huang et al., 2006).

Chemotaxis is the movement of PMN in response to chemoattractant gradients (Paape et al., 2003). Neutrophils migrate out through endothelial cells (diapedesis) while releasing matrix metalloproteinases to help break down the basement membranes and tissue stroma (Parkin et al., 2001; Paape et al., 2003). Chemoattractants induce an up-regulation of adhesion molecules on the surface of

vascular endothelium and PMN to aid in recruitment. Next the PMN are activated to move in the direction of the chemoattractant gradient (Parkin et al., 2001). One type of chemoattractant that is produced by the cell wall of bacteria is N-formyl-methionyl-leucylpheylalanine. This chemoattractant causes the mast cells to produce another chemoattractant, leukotriene B4, and macrophages to release IL-8. The chemoattractant C5a is generated as part of the complement pathway. Not only do chemoattractants cause PMN migration but they also up-regulate ICAM-1 found on endothelial cells which helps promote cell recruitment (Parkin et al., 2001). Both high and low concentration gradients of chemoattractants are capable of generating a response of PMN. Chemotaxis is the migration and polarization of PMN in response to a chemotactic gradient (Lokuta Et al., 2007). Chemotaxis has two possible processes. The first is actin independent sensing of chemoattractant gradients and the second is the actin mediated PMN polarization (Lokuta et al., 2007). This actin-dependent method uses a pseudopod that brings the PMN toward the chemoattractant. Neutrophils are easily activated, and when PMN are not round but polarized that indicates they are in their activated state.

1.2.3   Phagocytosis and Pathogen Destruction

During phagocytosis the PMN creates a membrane bound vesicle that contains the pathogen (Parkin et al., 2001). The PMN use pseudopodia to envelop the pathogen. These membrane bound vesicles fuse with the lysosome containing cytoplasmic granules forming phagolysosomes. Enzymes found in the granules include myeloperoxidase (**MPO**), lysozyme, cathepsins,alkaline phosphatase,ribo- and deoxyribonucleases, neutral proteases, elastases, etc.   (Parkin et al., 2001). The pathogens are then destroyed.

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Neutrophils can synthesizae and secrete ROS which can kill phagocytized pathogens (Mehrzad et al., 2002). Reactive oxygen species can kill both gram-positive and gram-negative bacteria (Mehzrah et al., 2002). Before ROS can be created the amount of oxygen taken into the cell first has to increase (Paape et al., 2003). The creation of ROS involves the reduction of oxygen by a membrane bound NADPH oxidase that is assembled de novo at the time of receptor activation. During internalization of the receptor bound agaonists (C3a, IgG, of IgMC3b opsoinized microbes, the NADPH is internalized into the phagosome and finally the phagolysosome vesicle (Parkin et al., 2001, Paape et al., 2003, Rinaldi et al., 2008). Phagosomes fuses with a lysosome during maturation to form a phagolysosome. The PMN first release superoxide and hydrogen peroxide. These ROS later interact on the phagosomal membrane to form hydroxyl radical and singlet oxygen (Parkin et al., 2001; Paape et al., 2003; Rinaldi et al., 2008). All of these ROS are powerful oxidants (Rinaldi et al., 2008).). The myeloperoxidase released into the phagolysosome catalyzes the halogenation of oxidant radicals to form hypohalous radicals using primarily Cl- and I-. Since myeloperoxidase adheres tightly to microbial surfaces, OCl- and OI- is formed directly on the surface of these microbes and triggers oxidation damage to microbial sturctures. These reactions kill microbes extremely fast in the phagolysosome.

1.3   The Dairy Industry- The Transition Period

The transition period for a dairy cow is between 2-3 weeks before calving until 2-4 weeks after calving (Sordillo et al., 1997; Mallard et al., 1998; Hammon et al., 2006; Rinaldi et al.,2008; Ingvartsen et al.,2012). This time is also called the

periparturient period and is a time when the cow will be going through metabolic, hormonal, and immunologic changes (Loiselle et al., 2009). This period of time is usually associated with a decrease in immune function for both innate and adaptive systems and an increase in susceptibility to metabolic disorders and chronic infections (Mallard et al., 1998; Rinaldi et al., 2008). The period causes physiological stress and is the time when the immune system is at its lowest function which makes a cow more prone to mastitis and metritis because both the phagocytic and the cytostatic functions of the PMN decrease (Mehrzad et al., 2002; Mallard et al., 1998; Ingvartsen et al., 2012). Many studies have shown that during this time there is a decrease in PMN migration, chemotactic ability, ROS production, and phagocytic activity (Rinaldi et al., 2008). Some studies also show a decrease in PMN ROS and MPO production (Mehrzad et al., 2002).

Almost all of the diseases of dairy cows including milk fever, ketosis, retained placenta, and displaced abomasum occur primarily in the transition period. Other diseases of dairy cows like laminitis and mastitis tend to develop most often around the same time period (Goff et al., 1997; Harp et al., 2005). There is also a greater chance for cows to develop sub-clinical infections during the transition period (Kimura et al., 2002). Endometritis is a reproductive disease caused by calving difficulties, retained placenta, and metabolic disorders (Kim et al., 2005). Studies show that after calving cows have a very high incidence of bacterial endometritis due to the decreased phagocytic and bactericidal activities of the PMN (Zerbe et al., 2000; Kim et al., 2005).

During the transition period the cow is in negative energy balance due to mobilization of body fat for energy (Loiselle et al., 2009). This means that blood

glucose levels are low and non-essential fatty acid (**NEFA**) levels are high. Reduced

dry matter intake (**DMI**) and increasing NEFA levels correlate with the decrease in

immune function during the transition period (Rukkwamsuk et al., 1999). Cows in

negative energy balance with high levels of beta-hydroxybutyric acid (**BHBA**)

develop more severe infections (Kremer et al., 1993). Another study found that a

decrease in PMN function is associated with negative energy balance and when too

much triacylglycerol accumulates in the liver (Zerbe et al., 2000).

1.3.1   Mastitis

Mastitis is the inflammation of the mammary gland and is currently both a

huge economic and animal health concern in the dairy industry (Sordillo et al., 1997;

Mallard et al., 1998; Scaletti et al., 2003; Stevens et al., 2011). Mastitis is one of the

only diseases found to show a relationship between milk yield and the disease, which

is why it is such an economic concern to the industry (Ingvartsen et al., 2012). The

reason that cows are more susceptible to mastitis during the periparturient period is

due to the decrease in PMN responsiveness during the transition period (Goff et al.,

1997; Mallard et al., 1998). There are many current ways of treating mastitis in the

dairy industry including using proper sanitation protocols, dry cow antibiotic therapy,

and decreasing exposure to environmental pathogens (Sordillo et al., 1997). However

even with these steps in play mastitis is still a growing problem affecting about 1/3 of

the cows (Sordillo et al., 1997; Scaletti et al., 2003). The mammary gland develops a

protective layer of keratin and lactoferrin during the dry off period that has anti-

microbial properties as part of the innate immune system (Goff et al., 1997). So far the

most common causes of intramammary infections (**IMI**) are streptococci,

staphylococci, and coliforms (Rowan et al., 2011). Environmental pathogens often

infect the mammary gland during the dry period when the cow is being held in poorer environmental conditions (Scaletti et al., 2003). During the dry period there is an increased incidence of IMI, but it usually does not result in mastitis. Only when lactation begins does clinical mastitis result from either the built up IMI or new IMI (Goff et al., 1997). Coliform mastitis is the main form of mastitis that occurs at this time (Goff et al., 1997). One study found that antibiotic therapy during this time is not helpful in treatment of coliform mastitis (Crist et al., 1992). Neutrophil ability to provide protection depends on their ability to perform chemotaxis, diapedesis, and phagocytosis (Mallard et al., 1998). For example, studies have shown that cases of mastitis are higher in cows with slow PMN, low ROS production and diapedesis (Sordillo et al., 1997; Mallard et al., 1998). Another study showed that the speed at which PMN could migrate out of blood affected the severity of *E. coli* mastitis (Mallard et al.,1998). Studies have shown that the severity of mastitis is directly related to how quickly the PMN and other leukocytes respond to the site of infection (Sordillo et al., 1997).

1.3.2   Feed Supplementations to Improve Immunity

Nutrition is important to consider when looking at the immune system (Wang et al., 2009). Researchers have found that many different kinds of feed additives can help improve the immune system and help reduce the spread of prominent diseases plaguing the industry, like mastitis (Rowson et al., 2010). Because mastitis is currently the second biggest cause of death in the dairy industry researchers are looking for a way to help lower the incidence of it and without the use of drugs. There is a growing concern in the industry that we are growing bacterial resistant strains of infections and this is driving producers to find alternative ways to prevent infection and boost

immune function. So far the use of antibiotics to treat IMI has been effective but antibiotics against *E.coli* and some other organisms are not as effective (Scaletti et al., 2003; Ryman et al., 2013), however it does not help prevent future IMI (Ryman et al., 2013). In order to prevent future IMI feed additives can be used to boost immunity. There is also a growing health concern about eating meat or dairy products that may contain antibiotic residues. For all these reasons feed supplements and vitamins and minerals are being utilized more and more to try and improve animal health through nutrition (Scaletti et al., 2003).

One feed additive that has been found to enhance the immune response is OmniGen-AF (Prince-Agri Products Inc., Quincy, IL), a proprietary supplement containing yeast and vitamin B complex that helps the immune system cope with pathogens (Ryman et al., 2013). Past studies have shown that this additive changes the response of PMN markers like L-selectin and IL-1β (Wang et al., 2009). Other studies also show that feeding OmniGen-AF reduces the incidence of mastitis on farms (Wada et al., 2008). One study used mice given a mastitis challenge to determine the effectiveness of OmniGen-AF on response to bovine origin *Staphylococcus aureus, Streptococcus uberis, Escherichia coli,* and *Klebsiella pneumonia* (Rowson et al., 2010). In this study OmniGen-AF reduced mastitis in mice induced by S. *uberis,* E. *coli*, and S. *aureus* (Rowson et al., 2010). They also found that when mice were fed OmniGen –AF there was an increase in the mammary tissue RNA concentration of both MPO and MHC. Myeloperoxidase is involved in generation of ROS which shows that inflammation and PMN migration into tissues increased with this feed additive, compared to cows not given any OmniGen-AF. Because MHC was also highly

enhanced, antigens were being presented to the adaptive immune system at a faster rate allowing for a better response time (Rowson et al., 2010).

Past studies have shown that feeding OmniGen-AF to immunosuppressed sheep increased the expression of both PMN proteins IL-1β and L-selectin. This allowed the PMN to migrate and function as they would in a healthy animal to control infection (Wang et al., 2009). Interleukin-1β is cytokine that functions to both increase endothelial permeability to proteins and help initiate the adaptive immune system. A study done by Wang et al., 2009 cows were fed OmniGen-AF. These cows showed a change in PMN life expectancy and cells went through apoptosis later compared with cows not given OmniGen-AF. This was shown by the change in IL-4 receptor expression (Wang et al., 2009). Cell communication also changed in the cows that were given this feed additive and was shown by measuring gene expression including angiopoietin-2 and interleukin converting enzyme (ICE). Several studies have been done on the effects of adding OmniGen-AF on animals diets. Another study found that cows supplemented with OmniGen-AF had PMN with a higher phagocytic activity and ROS production compared to those not given the additive (Ryman et al., 2013).

Other feed additives known to benefit the dairy cow's immune system are mineral and vitamin supplements. Minerals and vitamins are important to insure a properly working immune system (Andrieu, 2008). It has been shown that the major minerals and vitamins that can positively affect the immune system are selenium, vitamin E, chromium, cobalt, zinc, manganese, iron, copper, and vitamin A (Andrieu., 2008; Wang et al., 2009; Nemec et al., 2012). When an animal is deficient in one or more of these minerals and vitamins, both the innate and the adaptive immune response may be weakened (Nemec et al., 2012). Other studies indicated that the

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specific nutrients that are important to help lower incidences of mastitis are selenium, vitamin E, vitamin A, β-carotene, copper, and zinc (Sordillo et al., 1997; Scaletti et al., 2003).

Many of these nutrients benefit the immune system because their abilities act as antioxidants. Because immune cells create many ROS when they are active, antioxidants often become depleted (Wang et al., 2009). Reactive oxygen species production also increases during late gestation, parturition, and lactation (Spears et al., 2008). Free radicals created by oxidative reactions can cause damage to tissue throughout the body (Andrieu, 2008). When the production of ROS is greater than the amount of antioxidants present, oxidative damage occurs, leading to lipid peroxidation and cell death (Spears et al., 2008). Therefore creation of more antioxidants is essential for the proper function of the immune system. Membranes of most immune cells contain high concentrations of polyunsaturated fatty acids which makes them particularly susceptible to ROS-induced damage (Andrieu, 2008; Spears et al., 2008; Wang et al., 2009). When the generation of ROS exceeds antioxidant capacity an animal can become immunosuppressed (Andrieu, 2008).

Selenium is an important mineral for a functioning immune system because it is used in the creation of the antioxidant glutathione peroxidase enzymes (Sordillo et al., 1997; Spears et al., 2008). Glutathione peroxidases are a group of enzymes whose job is to catalyze the conversion of H_2O_2 to water and eliminate oxidant stress (Spears et al., 2008). Selenium supplementation leads to shorter mastitis duration and reduced severity. Selenium is also part of another antioxidant enzyme, thioredoxin reductase (Andrieu., 2008; Spears et al., 2008). Selenium deficiency has been shown to lead to

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decreased PMN function, especially a decrease in migratory ability (Spears et al., 2008).

Vitamin E is a lipid soluble vitamin and a main component in cellular membranes, preventing the damaging ROS from destroying membrane lipids (Sordillo et al., 1997; Spears et al., 2008). Vitamin E also appears to improve PMN function overall, including phagocytosis (Spears et al., 2008). Cows that were supplemented with vitamin E show decreased incidence of mastitis and increased neutrophil bactericidal ability (Sordillo et al., 1997; Spears et al., 2008). Vitamin A (precursor β-carotene) is found in cell membranes and can destroy free radicals (Sordillo et al., 1997). Both vitamin A and β-carotene have been shown to positively stimulate the immune system. Cows supplemented with vitamin A have been shown to have lower milk somatic cell count (**SCC**; Sordillo et al., 1997).

Copper plays an important role in the immune system by the creation of Cu dependent enzymes (Xin et al., 1991). Superoxide dismutase (**SOD**) is a Cu dependent antioxidant enzyme and many studies have reported relationships with a decrease in immune function when Cu in feed is low (Xin et al., 1991; Andrieu, 2008; Spears et al., 2008; Nemec et al., 2012). The function of SOD is to convert superoxide radicals to hydrogen peroxide in the cytosol (Spears et al., 2008). Copper is also an important element in the antioxidant serum protein ceruloplasmin (Sordillo et al., 1997; Spears et al., 2008). Both of these proteins are known for their strong antioxidant abilities. Cows with copper deficiency have been shown to have decreased PMN function. The study done by Xin et al. (1991) showed that SOD activity and intercellular Cu concentration in PMN decreased when Cu was low in the liver. This study also showed that low Cu decreased intercellular SOD activity and

PMN bactericidal ability. This suggests that low Cu levels may lower immune function. A study done by Scaletti at al. (2003) showed that Cu supplemented dairy cows had lower bacterial counts in milk, lower SCC, lower clinical udder score, and lower rectal temperature when challenged with intramammary *E coli*. Manganese is another element that is important for the function of SOD and is thought to have overlapping impacts on the immune system as Cu (Andrieu., 2008; Nemec et al., 2012).

Zinc is an important element in Zn-dependent SOD, an antioxidant enzyme (Sordillo et al.,1997; Andrieu., 2008; Spears et al., 2008; Nemec et al., 2012). Zinc is also a component in another antioxidant enzyme , metallothionein; this enzyme is a metal binding enzyme that binds hydroxyl radicals (Spears et al., 2008). Zinc is also thought to play a role in stabilizing the cell membrane from ROS species (Sordillo et al., 1997; Andrieu, 2008; Nemec et al., 2012). Cows that are zinc deficient are more prone to infection (Sordillo et al., 1997). One study showed the effect of Zn on beef cattle after exposure to bovine rhinotracheitis virus. Zinc seemed to improve immune function of the cattle and they recovered faster when supplemented with Zn; they also had a better rate of gain after recovery (Chirase et al., 1991)

1.4   Measuring Chemotaxis in the Lab

To study chemotaxis in the lab, blood is collected from the animal, treated with an anticoagulant, and PMN are isolated using lab specific protocols. There are two main platforms for chemotaxis assays, the transwell assay (Cell Biolabs, Inc., San Diego, CA) and NeuroProbe chamber assay (Neuro Probe, Inc., Gaithersburg, MD). In a transwell assay and NeuroProbe assay 2 chambers are separated by a membrane of different pore sizes (Lokuat et al., 2007). Chemotaxis can be measured by placing the

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chemoattractants only in the bottom. Neutrophils are placed on the top of the

chamber, and when the assay is finished the number of PMN that migrate though the

membrane is determined (Lokuat et al., 2007). The difference between the two assays

is that in a transwell assay you can only calculate the concentration of migrated PMN

and in a NeuroProbe you can calculate both adhered and migrated PMN.

1.4.1   Chemokineses

Chemokinesis, or random migration of PMN, can also be evaluated using these

two assay platforms. This is achieved by adding chemoattractants to both the top and

bottom chambers during a transwell or NeuroProbe assay (Lokuat et al., 2007).

Chemokinesis can be measured to determine loss of locomotive ability due to

abnormal adhesion molecules, receptor dysfunctions, or inability for cells to polarize

(Zigmond et al., 1986).

1.4.2   Laboratory Variation in PMN Isolation and Chemotaxis Assays

There are many different protocols followed by different labs for PMN

isolation and chemotaxis measurement (Table 1). Anticoagulants used when collecting

blood include EDTA, some heparin, and acid citrate dextrose. Bovine PMN are most

commonly isolated by spinning whole blood and lysing cells at bottom of the cell

pack, but differential centrifugation using a Percoll can be done for a more purified

PMN population. During the chemotaxis assays, different groups use membranes with

3 μm, 5 μm, or 8 μm pore size to separate the top and bottom chambers. Length of

chemotaxis incubation ranges from 30 to 120 min and though most incubations are

performed in incubators with 5% CO_2 / 95% air, some are performed with 10% CO_2 /

90% air.. Chemoattractants evaluated include IL-8, C5a, and zymosan-activated serum

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(zymosan activated serum is done to generate C5a and C5a desarg). Finally, chemotaxis is measured either by counting PMN adhered to the membrane or by measuring PMN concentration in the bottom wells of the chamber. It is likely that all of these procedural differences cloud our ability to compare results across different experiments.

1.5 CO_2 in Cell Culture Media

Many cell culture media use a bicarbonate based buffer system. A major component of these systems is $NaHCO_3$ which in solution dissociates to Na^+ and HCO_3^- (Shipman et al., 1969). HCO_3- is a buffer and in a water based media HCO_3^- is in equilibrium with H_2CO_3 and CO_2 and H_2O. When CO_2 is lost to the environment this causes an accumulation of OH^-, driving up the pH of the media. Therefore, media that use bicarbonate as the primary buffer need to be incubated in CO_2 to prevent CO_2 loss from the system. 5% CO_2 also mimics the normal conditions in the body which has also been used as a reason for its use in cell culture systems. HEPES is a buffer that does not depend on CO_2 (Shipman et al., 1969). Cells in media with HEPES as the primary buffer do not need to be incubated with CO_2.

1.6 CO_2 and pH Effect on PMNs

Few studies have evaluated the effect of CO2 during incubation on PMN activity, but Shimotakahara et al. (2007) found that incubation of PMN with 100% CO_2 inhibited chemokinesis and chemotactic movement and also resulted in a loss in ROS production ability. Presumably effects of CO_2 on PMN are due to effects on extracellular acidity which will decrease in response to increasing levels of CO_2.

Neutrophil function is affected by changes in both intracellular and extracellular pH. There are also some studies that show that the complement pathway is influenced by pH (Kellum et al., 2004). All cells control intracellular pH by using bicarbonate transport proteins including HCO_3^-/Cl^- anion exchange (Giambelluca et al., 2011). When PMN are activated by chemoattractants, they release superoxide, the HCO_3^-/Cl^- exchange protein is inhibited, and a Na^+/H^+ proton channel is opened, all of which cause their intracellular pH to drop significantly (Giambelluca et al., 2011). During the recovery period, PMN Na^+/H^+ proton channels are actively reversing this acidification to restore pH, but this can lead to alkalization (Giambelluca et al., 2011). During inflammation, lactate production and hydrogen efflux from surrounding cells reduces extracellular pH which enhances migration of PMN to that area (Zigmond et al.,1981; Trevani et al., 1999; Murata et al., 2009). When intracellular pH of PMN is reduced in response to a chemoattractant and extracellular pH is reduced in response to inflammation, this enhances PMN adhesion to the vascular endothelium, increasing the ability of PMN to reach the site of infection (Serrano et al., 1996). However, if either extracellular or intracellular pH becomes too acidic, this can affect cellular functions including ion transport activities, enzyme activities, DNA synthesis, ROS production, cytokine production, and cAMP and calcium regulation (Lardner., 2001; Murata et al., 2009).

Studies have also been conducted to evaluate the effect of pH on PMN function in vitro. When pH rises above 7.6 there is a decrease in chemotaxis (Lardner, 2001). The optimal pH for PMN migration has been found to be between 6.8-7.6, with a peak migration rate at 7.4 (Bryant et al., 1966; Nahas et al.,1971). When leukocyte motility was measured, movement was impaired when pH was below 6.5. Another

study showed a complete loss in chemotaxis movement at pH 6 and an increase in chemokenisis (Lardner., 2001). Other studies have shown that there is an increase in migration to more acidic pH (5) than compared to basic pH (8) in a pH gradiant (Zigmond et al., 1981). This study showed that PMN move more rapidly to a pH of 5.7 (Zigmond et al., 1981). One study has shown that leukocyte chemotaxis, is negatively affect when extracellular pH drops between 5.5-6 (Kellum et al., 2004). Early studies have shown that acidic pH increases PMN movement (Nahas et al., 1971).

Changes in pH also affect other aspects of PMN function. An acidic extracellular pH (5.5 to 6.0) has been found to decrease phagocytosis and increase in apoptosis (Kellum et al., 2004; Mills et al., 2006). Effects of acidity on PMN ROS production have been more variable, with some studies finding that ROS production increases in response to reduced pH while other studies have found impaired ROS production (Trevani et al., 1999; Lardner., 2001; Kellum et al., 2004; Mills et al., 2006).

1.7 Objective

The objective of this experiment was to determine the impact of incubation with or without CO_2 and with or without HEPES on bovine neutrophil chemotaxis.

1.8   Hypothesis

We hypothesize that incubation with CO_2 will reduce media pH and negatively affect PMN chemotaxis and that inclusion of HEPES in the assay media will ameliorate this effect.

Chapter 2

Materials and Methods

2.1 Blood Collection and Neutrophil Isolation

On each sampling date, blood was collected from four lactating cows (4-163 DIM; n=12 cows total; Figure 1). Blood was drawn from the jugular vein into 3 blood tubes per cow containing EDTA and immediately placed on ice after collection. The 3 blood tubes from each cow were then combined into one 50 mL conical tube that was then centrifuged at 1000×g for 45 minutes at 4°C.

The plasma layer, buffy coat and 2/3 of the red cell pack were removed and the erythrocytes were lysed by adding 12 ml of 4°C hypotonic lysing solution (10.6 mM Na_2HPO_4, 2.7 mM NaH_2PO_4) and mixing for 40 seconds. To restore the isotonicity to normal, 6 ml of 4°C hypertonic restoring solution (10.6 mM Na_2HPO_4, 2.7 mM NaH_2PO_4, 430 mM NaCl) was added to each tube. The tubes were centrifuged again at 800×g for 5 min at 4°C, supernatant was decanted and 10 ml of Hank's Balanced Saline Solution (**HBSS**; Mediatech Inc., Manassas, VA) was added to each tube. The tubes were centrifuged again, supernatant was decanted and 10 ml of HBSS was added to each tube. The supernatant was decanted again and 2 ml of assay media (HBSS with 5% fetal bovine serum (FBS) either with or without 10 mM HEPES) was added to the tubes. pH of stock HEPES was measured to ensure it was at 7.4.before incubation.

Neutrophils (PMN) were re-suspended into assay media with a Pasteur pipette to break up the pellet that formed. Neutrophils remained on ice throughout the experiment.

2.2   Measuring Neutrophil Concentration

To measure PMN concentration, 15 μl cell suspension was added into each of 2 duplicate cups containing 10 ml of isotone solution (Beckman Coulter Inc., Fullerton, CA). Two to three drops of red cell lysing agent Zap-OGLOBIN (**Zap**, Beckman Coulter, Inc.) were added to both cups and the cups were inverted three times to ensure cells were evenly dispersed. Neutrophil concentration was determined using a Coulter Counter (Beckman Coulter Inc.) using the average of 2 counts per sample. The concentration was adjusted to 2×10^6 cells/mL in assay media (Figure 1).

2.3   Using the Chemotaxis Chambers

Four 48 well chemotaxis chambers (Neuro Probe Inc., Gaithersburg, MD) were used throughout the experiment (Figure 2). Two chambers were used for incubations without HEPES and two were used for incubations with 10 mM HEPES. pH was measured in both the media with HEPES and media without after incubation took place. The bottom wells of the chambers contained 28 μl of media (with or without HEPES) supplemented with either 100 ng/mL of interleukin-8 (IL-8) or 50 ng/mL of complement component 5a (C5a).

A five μm polycarbonate membrane (Neuro Probe Inc., Gaithersburg, MD) was used to separate the upper and lower wells in all the assays. Once the bottom wells were filled, chambers were incubated at 37°C with or without the addition of 5% CO_2 for 10 minutes for the purpose of wetting the membrane. The top wells of the chamber were then filled with 50 μl of PMN suspension (Figure 1). Negative controls contained media alone in the bottom wells (with or without 10 mM HEPES) while positive controls contained 50 ng/mL of C5a in both top and bottom wells (with or without HEPES).

Each treatment combination was performed in triplicate wells. The chambers were incubated for 60 minutes at 37°C. Two chambers (one with HEPES and one without HEPES) were incubated in the presence of 5% CO_2 and the two remaining chambers were incubated in the absence of CO_2 (Figure 1).

2.4   Dyeing the Membrane

After incubation, cell suspension was pipetted out of the top wells of each chamber and collected into four separate 15 mL conical tubes (1 for each chamber) for determination of pH. The membrane was carefully removed and dipped in phosphate buffered saline (PBS) and non-migrated cells were scraped off. After excess cells were scrapped off the membrane was dipped into methanol , xanthene dye , and thiazine dye (HEMA 3 Stain Set; Fisher Scientific, Kalamazoo, MI). Excess dye was washed away with distilled water and the membrane was placed on a glass slide to dry.

2.5   Neutrophil Migration

Neutrophil migration was determined using two methods: adherence to the membrane and migration to the bottom wells of the chemotaxis chamber (Figure 1). Adherence to the membrane was counted using a microscope under a 40x objective and average adherence in 5 random fields/well was recorded. Migration of neutrophils to the bottom wells was determined by measuring PMN concentration. Cups were filled with 10 ml of isotone solution and 15 μl of cell suspension from the bottom wells was placed in each cup. Two to three drops of Zap was added to each cup and PMN concentration was determined using a Coulter Counter. Blanks were ran approximately every 16 cups, and the average of blank measurements was used to adjust the PMN concentration.

2.6   Calculations

Adherence (**Ad**) was calculated as the average adherence to the bottom of the membrane across the five fields per well in the three replicate wells. Relative adherence (**RelAd**) was then found by dividing Ad of the test samples by Ad of that cow's negative control sample. Concentration (**Con**) was the average concentration of migrated PMN measured in the bottom wells across the three replicate wells. Relative concentration (**RelCon**) was calculated by dividing Con of test samples by Con of that cow's negative control sample. Data was analyzed with the Glimmix procedure of SAS with the fixed effects of HEPES, CO_2, and chemoattractant and all interactions and random effects of date and cow within that date. Data for RelAd were log transformed prior to analysis in order to obtain homoegeneity of residual variance.

Chapter 3

RESULTS

3.1 Average pH

The average pH for assay media when both CO_2 and HEPES were not present was 7.64 (Figure 3). When no CO_2 was present but HEPES was the average pH was 7.90. When CO_2 was present but HEPES was not the average pH was 7.37. When both CO_2 and HEPES were present the average pH was 7.45.

3.2 Chemoattractants on Neutrophil Migration

Main effects of chemoattractant, CO_2, and HEPES for the four variables are shown in Tables 1-3, respectively. Chemotaxis toward IL-8 and C5a was higher than toward controls for all four measures (Table 2). Chemotaxis toward IL-8 was not different from chemotaxis toward C5a across all four measures. For Ad, there was no difference between migration to positive and negative control wells. For Con, migration to the positive control was intermediate between migration to the negative control and migration to C5a and IL-8.

3.3 CO_2 on Neutrophil Migration

Chemotaxis of PMN was affected by the presence or absence of CO_2 during incubation for 3 of the 4 measures (Table 3). For Con the presence of CO_2 during incubation increased PMN migration, but there was no difference for RelCon. For both Ad and RelAd CO2 decreased adhesion to membrane.

3.4 HEPES on Neutrophil Migration

Chemotaxis of PMN was affected by the presence of HEPES for both Con and RelCon (Table 4). For Con the presence of HEPES increased migration of PMN, but for RelCon was the presence of HEPES decreased PMN concentration. There was no effect of HEPES on Ad or RelAd.

3.5 Interaction of HEPES and CO_2

Interactions among main effects are shown in Figures 4-8. There was a trend for an interaction between HEPES and CO2 on Con of migrated PMN (P=0.06; Figure 4). When CO2 was present HEPES had no effect on concentration of PMN. However when CO_2 was not present HEPES increased the concentration of PMN.

There was a trend for an interaction between HEPES and CO2 on RelCon of migrated PMN (P=0.08; Figure 5). When CO_2 was present HEPES decreased the concentration of PMN. However when CO_2 was not present HEPES had no effect on the RelCon of PMN.

There was an interaction between HEPES and CO_2 on Ad of migrated PMN (P=0.0001; Figure 6). When CO_2 was present HEPES increased Ad of PMN. However when CO_2 was not present there was no effect of HEPES on Ad of PMN.

3.6 Interaction of HEPES and Chemoattractants

There was an interaction between HEPES and chemoattractant on Con of migrated PMN (P=0.002; Figure 7). When HEPES was present there was a numeric increase in PMN migration to negative control, C5a, and positive control wells compared to when HEPES was not present. However, this HEPES-induced increase was significant for migration of PMN to IL-8.

3.7 Interaction of CO_2 and Chemoattractants

There was a trend for an interaction between CO_2 and chemoattractant on Ad of migrated PMN (P=0.02; Figure 8). When CO_2 was absent, Ad in response to both IL-8 and C5a was greater than both the negative and positive controls. When CO_2 was present there was no significant difference among the four chemoattractant treatments.

Chapter 4

DISCUSSION

Chemotaxis toward IL-8 was not different from chemotaxis toward C5a across all four measurements (Table 2). Currently, most research does not directly compare migration to different chemoattractants. C5a binds to its receptor C5aR found on the plasma membrane of leukocytes (Woodruff. 2001). The C5aR receptor is a G- coupled protein receptor, which is made up of seven helically shaped transmembrane stretches, an N- and C- terminal fragment, and external and internal loops (Woodruff., 2001; Nikiforovich., 2008).The first binding site for the C5aR receptor is found at the N-terminal side of the G protein (Nikiforovich., 2008). IL-8 binds to its receptor CXCR1, another G-coupled protein receptor found on the cell membrane of PMN (Sarmiento., 2008).IL-8 binds to the N- terminus of the G- coupled membrane bound receptor (Park., 2011). The majority of G-coupled receptors are composed of the seven transmembrane segments connected to three extracellular and three cytoplasmic loops (Sarmiento., 2008). These G- coupled proteins also consists of an extracellular N terminal and an intercellular C- terminal (Sarmiento., 2008). The similarities in the structure of the G- protein receptors and the N-terminal binding sides could lead to similar actions of PMN to the two chemoattractants evaluated in this study.

For Con the presence of CO_2 during incubation increased PMN migration, but for Ad CO_2 decreased adhesion to membrane (Table 3). In the study done by Shimotakahara et al., 2007, experimenting with different levels of CO_2, PMN migration was determined by adhesion to a membrane. They found that there was a pH

drop to about 6.2 when the chambers were incubated in 100% CO_2. They also observed no chemokinesis of PMN in the chambers incubated in 100% CO_2, or chemotaxis due to IL-8 (Shimotakahara et al. 2007). While we only used 5% CO_2, there was still a decrease in Ad to the membrane when CO_2 was used. However, when Con was measured PMN migration increased in CO_2. In our experiments, it is possible that CO_2 may have increased migration to the bottom wells of the chamber but decreased expression of adhesion factors.

CO_2 decreased media pH, while HEPES increased pH (Figure 3).Across the different dates, pH was more variable when HEPES was included then when HEPES was not included. Overall in CO_2, both with and without HEPES, pH was closest to physiological pH. Without CO_2 pH was more alkaline than desired. The pH for the best PMN migration was been shown to be between 6.8 and 7.6 (Bryant et al., 1966). In a study done by Rabinovitch et al. (1979) a drop in migration is observed when pH goes above 7.6. Another study also reported complete loss of movement at pH 7.9 and above (Lander, 2001). The average highest pH that was found in this study was 7.9, when CO_2 was absent but HEPES was present (Figure 3). When looking at the effect of HEPES (Table 4) there was not effect of HEPES on Ad or RelAd and inconsistent effects of HEPES on Con and RelCon. This lack of effect could be due to undesirable media pH when HEPES is present. The presence of CO_2 increased Con and decrease Ad and RelAd (Table 3). The reason for this is unclear but perhaps CO_2 decreased expression of adhesion molecules.

The effects of HEPES on chemotaxis were dependent on CO_2. When CO_2 was present HEPES had no effect on the Con of PMN, but when CO_2 was not present HEPES increased the presents of PMN (Figure 4). When CO_2 was present HEPES

increased Ad of PMN (Figure 6).When CO2 was present HEPES decreased the concentration of PMN when measuring using RelCon (Figure 5). However when CO_2 was not present HEPES had no effect on the RelCon of PMN. CO_2 was expected to cause a negative effect on chemotaxis and HEPES was expected to alleviate this effect. This was because we expected CO_2 to result in acidic pH and that HEPES would restore physiological pH. However, physiological pH was only obtained in the treatments with CO_2. The most alkaline pH was found in the absence of CO_2 and the presents of HEPES. Therefore based on pH data alone we would expect an inhibition in chemotaxis (Lardner. 2001). However, this treatment combination never resulted in the lowest chemotaxis and treatment combinations that decreased chemotaxis were different for each of these three measures. Further replication would be useful. However based on our results we think if HEPES is used it should be incubated with 5% CO2.

CO2 and HEPES are both important for detecting treatment differences for Con and Ad. For Con when HEPES was not present there was a decrease in the difference between the treatmetns IL-8 and C5a compared to the controls (Figure 7). For Ad, the presence of CO_2 also reduced the difference between the tretments IL-8 and C5a compared to the controls (Figure 8). Both of these results support the hypothesis that CO_2 will inhibit PMN chemotaxis and that HEPES will inhance PMN chemotaxis. However this treatment combination resulted in the most alkaline pH (Figure 3) which would be expected to cause a loss in PMN function (Larnder, 2001).

The two major platforms for measuring PMN chemotaxis are NeuroProbe chambers and Transwell systems. Chemotaxis in NeuroProbe chambers can be measured either by counting PMN adherence to the membrane or by PMN

concentration in the bottom wells. For the Transwell systems, only PMN concentration in the bottom wells can be measured. One of our objectives was to compare migration measured as the amount that migrated thought the membrane (Con and RelCon) verses counting the amount of PMN adhered to the membrane (Ad and RelAd). The results suggest that data interpretation will differ depending on data collection method. It is unclear from our data which measurement method is most appropriate and this lack of consistency may help to explain why conclusions often vary among research groups when evaluating the same treatments.

Our studies included chemokinetic controls that contained chemoattractant in both the bottom and the top wells of the chamber. These controls were used to determine how much of the PMN movement was due to random migration compared to response to the chemoattractant gradient. Our chemokinetic controls (labeled as + control in figures 7 and 8) never differed from the negative control wells that contained no chemoattractant in either chamber. This suggests that random migration was not affected by the CO_2 or HEPES treatments. This was counter to our hypothesis that CO_2 might cause activation of PMN, which in turn would lead to an increase in chemokinesis.

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Appendix A

TABLES

Table 1: Variations in protocols followed by for PMN isolation and chemotaxis measurement.

Variant	Method A	Method B	Method C	Method D
Anticoagulant	EDTA (1, 5, 8, 9)[1]	Heprin (3, 6)	Acid citrate dextrose (4, 11)	------------
PMN separation	No differential centrifugation (1, 5, 6, 8, 9, 10)	Percoll gradient (11)	------------	------------
Membrane pore size	3 µm (2, 3, 7, 8, 12)	5 µm (4, 11, 10)	8 µm (9)	------------
Incubation time	30 min (3, 4, 11)	60 min (6, 10)	90 min (8, 9, 12)	120 min (7)
Incubation conditions	5% CO_2/95% air (2, 3, 4, 5, 8, 9, 12, 11, 10)	10% CO_2/ 90% air (6)	------------	------------
Counting method	PMN adhered to membrane (3,4,5, 11)	PMN concentration in bottom chamber (8, 9, 10, 12)	------------	------------
Chemoattractant	Both IL-8 and C5a (5, 10)	IL-8 only (7, 11)	Zymosan-activated serum (12)	------------

[1]Studies compared are identified by the following numbers: 1 = Carlson et al., 1973 (cows); 2 = Mulder et al., 1993 (sheep); 3 = Sugawara et al., 1995 (humans); 4 = Kimura et al., 2002 (cows); 5 = Auchtung et al., 2004 (cows); 6 = Lokuat et al., 2007 (mice); 7 = Shimotakahara et al., 2007 (Human); 8 = Loissele et al., 2008 (cows); 9 = Stevens et al., 2011 (cows); 10 = Nemec et al., 2012 (cows); 11 = Revelo et al., 2012 (cows); 12 = Ster et al., 2012 (cows)

Table 2: Main effects of chemoattractants on PMN migration

Measure[1]	Chemoattractant				SED	*P*-values
	-Control	IL-8	C5a	+Control		
Con, cells/mL	928,698^c	1,898,820^a	1,705,771^a	1,280,284^b	117,202	<0.0001
RelCon, ratio to control	-	2.47^a	2.26^a	1.63^b	0.18	<0.0001
Ad, cells/5 fields	47.43^b	84.47^a	74.18^a	47.37^b	6.69	<0.0001
RelAd, ratio to control, log$_{10}$	-	0.3132^a	0.2358^a	0.0173^b	0.0778	0.0002
RelAd, Back Transformed	-	2.0568	1.7211	1.041	1.1962	-

[a-c] means in columns with unlike superscripts are different between treatments (*P*<.0001)

[1] Concentration (**Con**) was the concentration of migrated PMN measured in the bottom wells. Relative concentration (**RelCon**) was calculated by dividing Con of test wells by Con of negative control wells..Adherence (**Ad**) was the number of PMN per microscope field that where adhered to the bottom of the membrane. Relative adherence (**RelAd**) was then found by dividing Ad of test wells by Ad of negative control wells.

Table 3: Main effect of CO2 on PMN migration

Measure[1]	- CO_2	$_+ CO_2$	SED	*P*-values
Con, cells/mL	1,267,913[b]	1,638,874[a]	107,009	0.0008
RelCon, ratio to control	2.14	2.10	0.21	0.86
Ad, cells/5 fields	70.67[a]	56.06[b]	6.59	0.03
RelAd, ratio to control $\log_{10}$	0.3183[a]	0.0362[b]	0.0878	0.002
RelAd, Back Transformed	2.0811	1.0869	1.2240	-

[a-b] means in columns with unlike superscripts are different between treatments (*P*<.03)
[1] Adherence (**Ad**) was calculated by counting PMN that where adhered to the bottom of the membrane. Five fields per well were counted to determine the average Ad value. Relative adherence (**RelAd**) was then found by dividing Ad test wells by Ad negative control wells Concentration (**Con**) was the concentration of migrated PMN measured in the bottom wells. Relative concentration (**RelCon**) was calculated by dividing Con test wells by Con negative control wells.

Table 4: Chemotaxis chambers contained media supplemented with or without HEPES

Measure[1]	- HEPES	+ HEPES	SED	P-values
Con, cells/mL	1,267,779[b]	1,639,007[a]	82,875	<0.0001
RelCon, ratio to control	2.28[a]	1.96[b]	0.15	0.03
Ad, cells/5 fields	62.13	64.60	4.73	0.60
RelAd, ratio to control log10	0.1739	0.1806	0.0633	0.92
RelAd, Back Transformed	1.4925	1.5157	1.1569	-

[a-b] means in columns with unlike superscripts are different between treatments (P<.03)
[1] Adherence (**Ad**) was calculated by counting PMN that where adhered to the bottom of the membrane. Five fields per well were counted to determine the average Ad value. Relative adherence (**RelAd**) was then found by dividing Ad test wells by Ad negative control wells Concentration (**Con**) was the concentration of migrated PMN measured in the bottom wells. Relative concentration (**RelCon**) was calculated by dividing Con test wells by Con negative control wells.
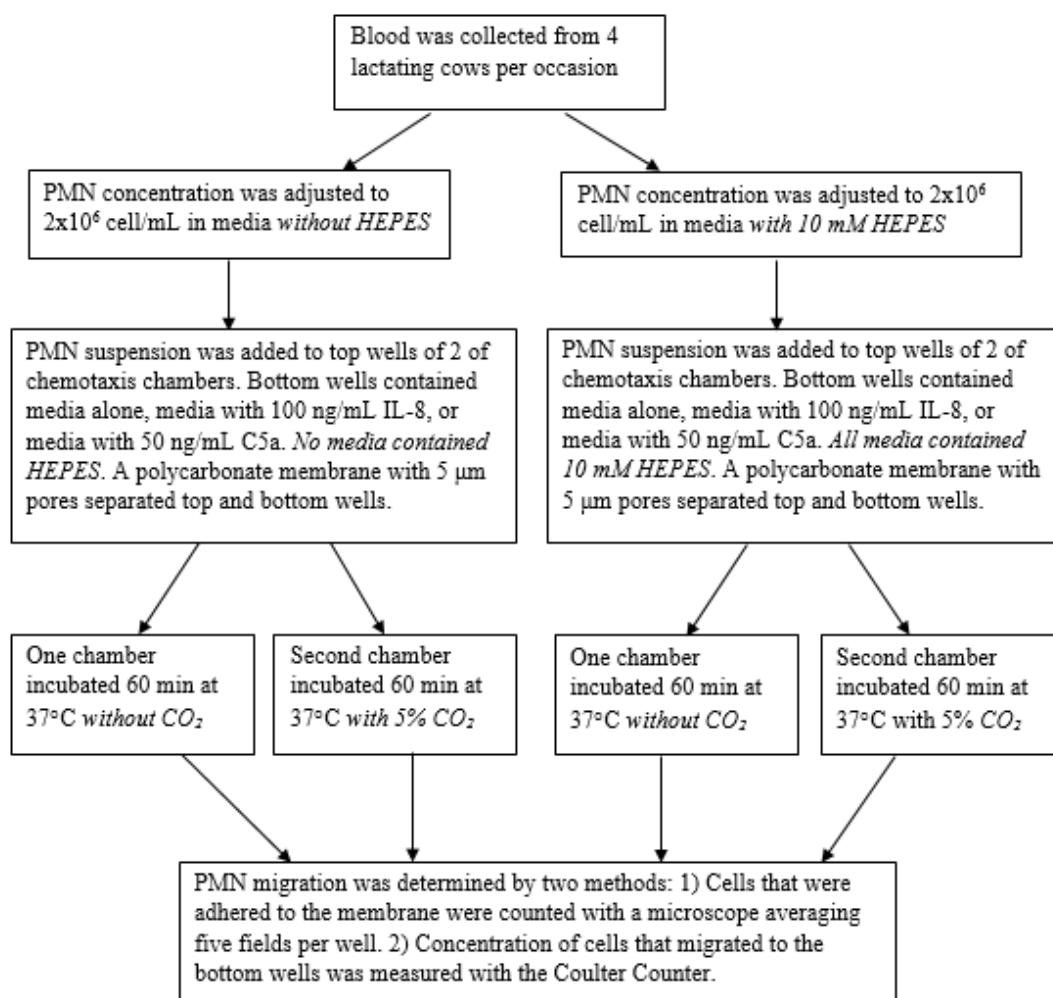
FIGURES

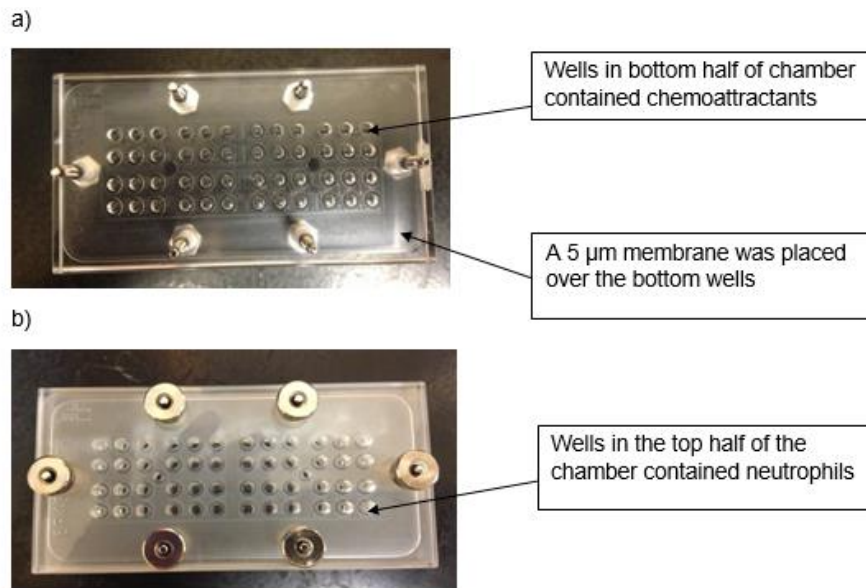Figure 1: Flow Chart of Experimental Procedures

Figure 2: Bottom portion of a chemotaxis chamber (a) and assembled chemotaxis chamber (b).
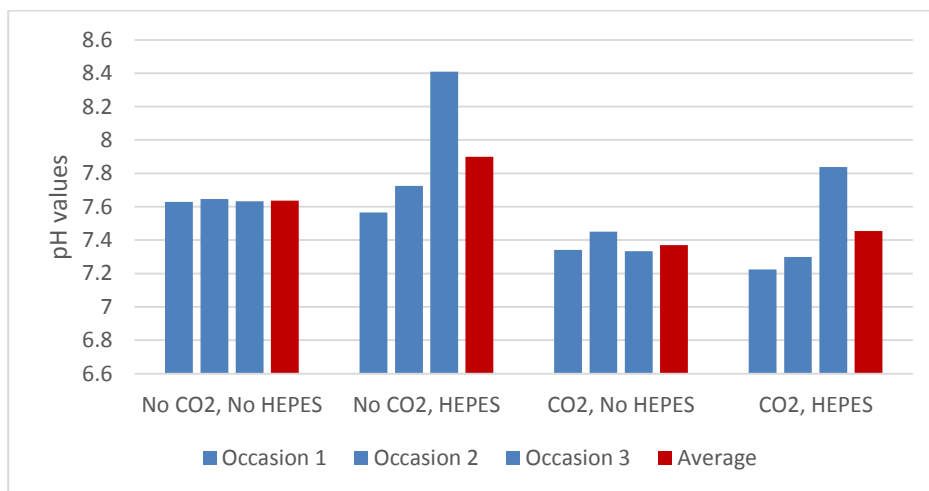

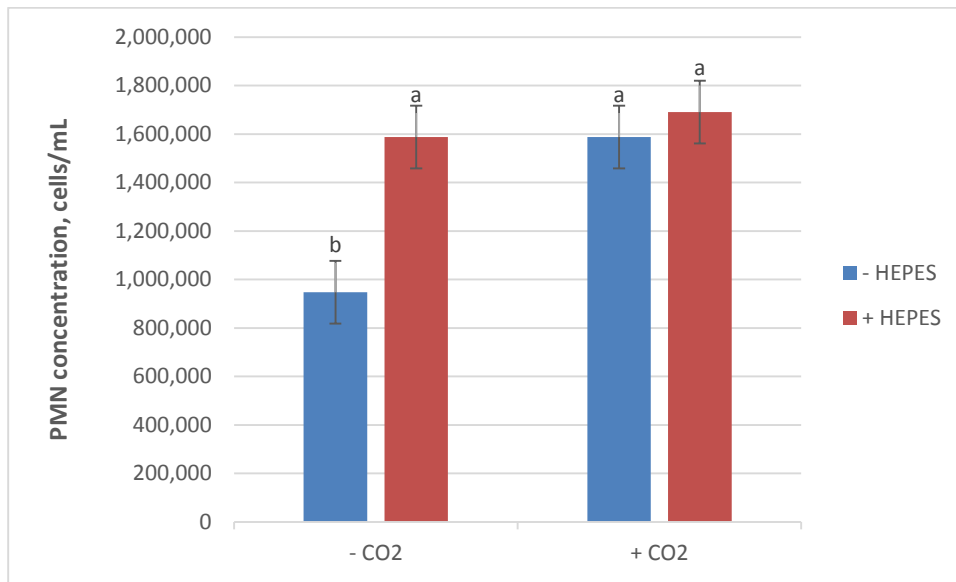Figure 3: pH measurements of PMN suspension following 1 hour incubation

Figure 4: Interaction of HEPES and CO_2 on concentration of migrated PMN (Con) measured in the bottom wells of chemotaxis chambers ($P = 0.06$).
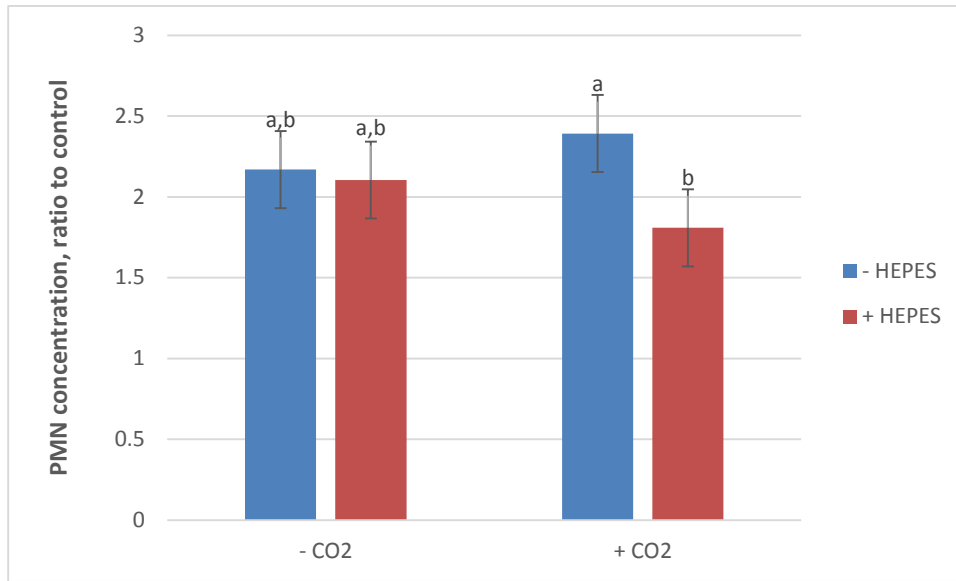
Figure 5: Interaction of HEPES and CO2 on relative concentration of migrated PMN (RelCon) measured in the bottom wells of chemotaxis chambers (P=0.08)
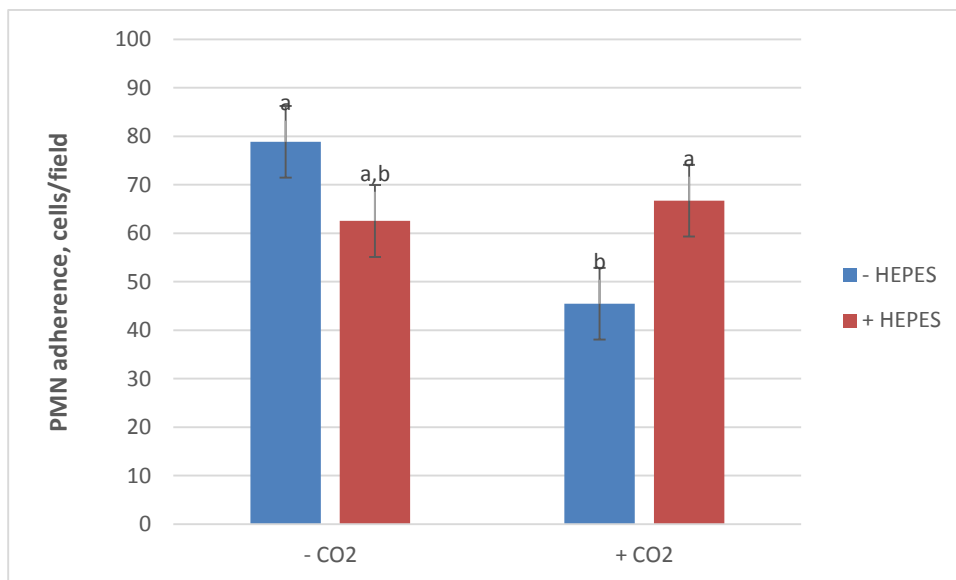

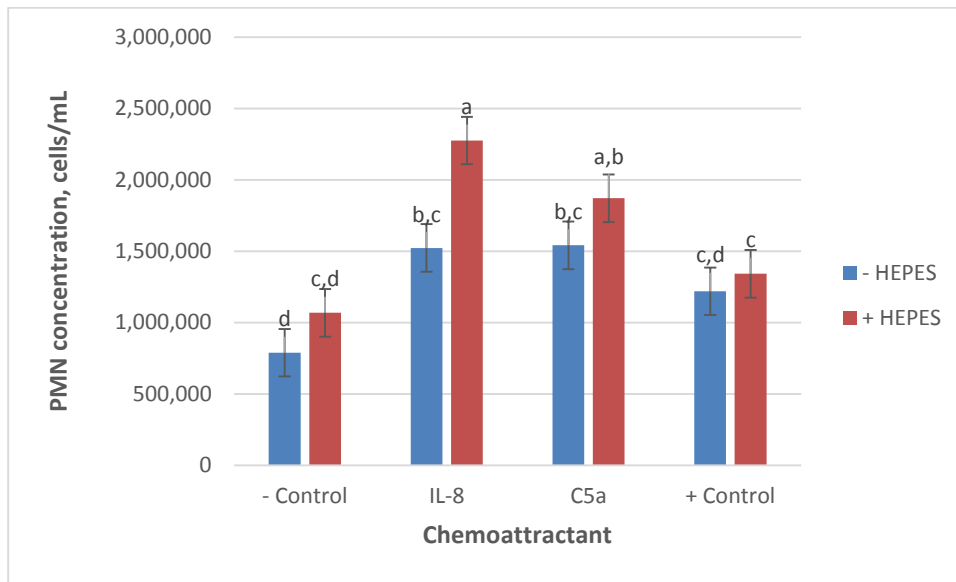Figure 6: Interaction of HEPES and CO_2 on PMN adhered (Ad) to membrane of chemotaxis chamber (P=0.0001)

Figure 7: Interaction of HEPES and Chemoattractants on concentration of migrated PMN (Con) measured in bottom wells of chemotaxis chamber (*P*=0.002)
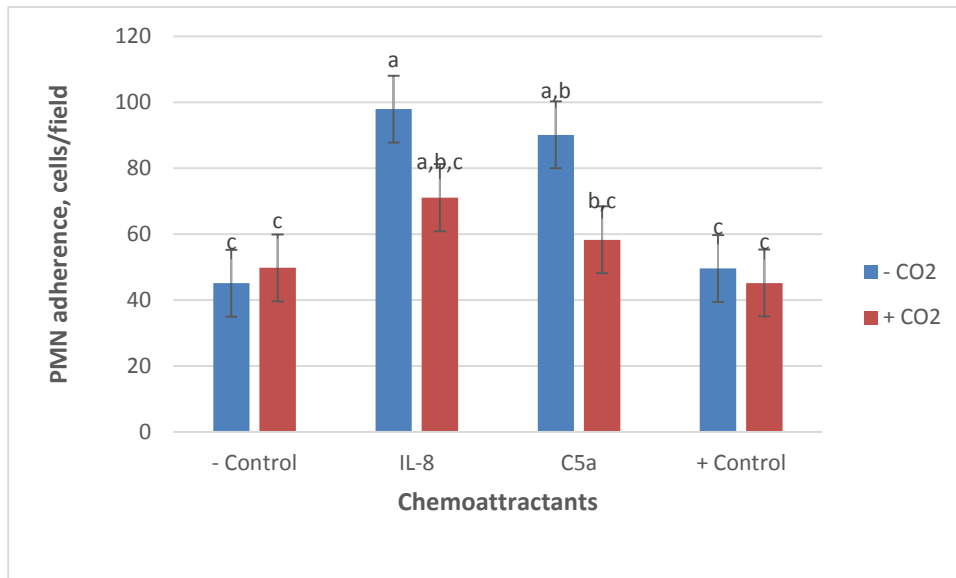
Figure 8: Interaction of CO_2 and chemoattractants on PMN adhered (Ad) to membrane of chemotaxis chamber (P=0.02)