SERUM TOTAL CALCIUM CONCENTRATION IN HOLSTEIN DAIRY BULLS DURING THEIR FIRST MONTH OF AGE: RELATIONSHIP WITH INFECTIOUS DISEASES AND IMMUNE FUNCTION

By

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To Felisa and Alex

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The health of the dairy calf is an important welfare issue. Farmers who are dedicated to the difficult task of raising calves are encouraged to implement several management programs to achieve their goals of a successful business while providing a good environment in which the animals live.

During the last decades several factors related to disease incidence in dairy calves have been investigated, resulting in new knowledge of how disease can be avoided or reduced. Some of these already known factors are the importance of the passive immunity provided by colostrum, good sanitation practices, immunization and nutritional programs. Besides all these well known factors, there is still a high incidence of disease in pre-weaned dairy calves, mostly due to digestive, respiratory, navel and joint diseases.

In the present study, the importance of calcium in blood in immune system function in the neonatal calve has been investigated. Disease incidence, phagocytic cell activation and cytokine production were measured comparing calves with different concentration of calcium in their blood.

Some trends of association, although not significant, were found between calcium concentration and incidence of respiratory infection and otitis media, and between calcium and cell activation at two days of age. These results encourage more study of the impact that calcium has in the immune response of the dairy calf and the incidence of disease in this group of animals.

CHAPTER 1
INTRODUCTION

Raising dairy calves can be a difficult task for dairy farmers because of a number of diseases that occur in the newborn calf that are of lesser importance to adult animals.

The incidence of disease is greatest in the first month of life, mainly due to the naïve immune status of calves (Barrington and Parish, 2001, Gulliksen et al., 2009). When calves go from the aseptic in utero environment to a highly contaminated environment on the farm, they are challenged with several pathogens against which they have yet to mount an immune response. This is the reason why it is of primary importance to keep the environment as clean as possible and to assure good transfer of passive immunity (maternal antibodies and leukocytes) through colostrum, to help fight those pathogens, at least until they are able to build a protective immune response against them.

Diseases affecting calves are important due to the economic losses associated with treatment and death (Tozer and Heinrichs, 2001). Suffering from diseases is also an important animal welfare issue. Several factors were found to be related with morbidity and mortality in dairy heifers, including those factors related with the calving process, colostrum management and farm characteristics, such as facilities, management, farm location and farm size (Trotz-Williams et al., 2007). Mortality and disease incidences have been reported in several studies; the most commonly reported causes of disease and death being neonatal diarrhea, septicemia, pneumonia, navel infection and arthritis. In the preweaning period scours and septicemia are considered the main problems and after being weaned, pneumonia is the most commonly reported disease (Gulliksen et al., 2009, Svensson et al., 2003, Wells et al., 1997).

Prevention of disease in the calfhood period should be the goal of any producer. Once the calf is sick, treatment may fail because the selected treatment may not be the most appropriate

for the etiologic pathogen or it may be initiated too late in the course of the disease (Lorenz and Vogt, 2006, Mechor et al., 1988, Vogel et al., 2001).

Preventive practices have been historically related with sanitation, colostrum and nutritional management, vaccination protocols and early detection and treatment of animals with signs of disease. Now, in the age when molecular technology is highly developed, genes related to disease resistance are being investigated for various diseases (Barthel et al., 2000, Bermingham et al., 2009, Zhang et al., 2007). Therefore it is worthwhile to investigate individual animal factors that predispose a population group to be more susceptible to infections compared to the general population.

The immune system is a complex network of interactions and ionized calcium has been identified in numerous immunologic processes as a second messenger in cell activation. Cell activation following pathogen recognition produces changes in the concentration of intracellular calcium following several complex enzymatic reactions. The increase in intracellular calcium is responsible for the activation of several transcription factors of various immunomodulatory peptides and it induces degranulation of certain cell types (Di Sabatino et al., 2009, Feske, 2007, Yu and Czuprynski, 1996).

The main objective of this thesis research is to determine if serum calcium concentration in the neonatal calf, measured as serum total calcium, is associated with disease in the preweaning period. A second objective is to determine if serum total calcium concentration alters the response of the immune system to selected stimuli.

CHAPTER 2
LITERATURE REVIEW

Calcium

Calcium (Ca^{2+}) is an element with atomic number 20 and 40.087 g/mol of atomic mass, belonging to the category of alkaline earth metals. In the mammalian system, calcium is involved in a great number of physiologic processes, as well as being an important part of the bone matrix. Some processes in which calcium is involved are blood coagulation, muscle contraction and nervous impulse transmission (Guyton and Hall, 2006).

Calcium in Blood

Calcium in blood plasma is present in three fractions, each being in equilibrium with one another. These fractions are the diffusible or ultrafiltrable fraction, comprised of ionized (iCa) and complexed calcium, and the non-diffusible calcium which is bound to plasma proteins. About 40% of the total calcium (tCa) is bound to plasma proteins, mainly albumin, and is not physiologically important. (Kanis and Yates, 1985, Kogika et al., 2006, Wills and Lewin, 1971).

Complexed calcium is usually bound to phosphate, lactate, sulfate, bicarbonate and citrate, and represent about a 10% of the total (Kanis and Yates, 1985, Kogika et al., 2006). Ionized calcium is the fraction considered biologically active and comprises half of the total calcium (Kanis and Yates, 1985). Therefore, clinically, it is considered the fraction of greatest importance.

Factors that affect calcium values in blood: In veterinary medicine, calcium status is often assessed using serum total calcium concentration, despite the fact only ionized calcium is biologically active. Improved methodologies for the measurement of iCa are becoming more readily available, although its use can be still a challenge in farm settings. A number of factors,

including blood pH and protein concentration, can affect the total and ionized calcium concentrations.

Calcium ion is bound by protein in the blood, occupying the space between spatially neighboring pairs of carboxyl groups in the protein molecule. The effect of pH upon ionized calcium in protein-containing fluids is due to the change in availability of carboxyl groups of proteins (McLean, 1934). In metabolic acidosis, H^+ radicals do not dissociate easily from the carboxyl groups and therefore there is less opportunity for calcium to bind to albumin. This results in an increase in the ionized calcium fraction (Kogika et al., 2006) without affecting the total calcium concentration (Kanis and Yates, 1985) (Figure 2-1).

Another difficulty found when measuring total calcium concentration includes the effect of abnormal plasma protein concentration. For example, hyperproteinemia can be induced by applying a tourniquet. The increase in plasma protein concentration due to capillary permeability after venous occlusion causes binding of calcium to retained proteins. This produces a more concentrated protein-bound fraction, corresponding to an increase in total calcium but not affecting ionized calcium (Berry et al., 1973) (Figure 2-2). They estimated a change in human plasma total calcium of 0.091mg/100ml for every 0.1g/100ml change in serum albumin, and recommended that total calcium should be corrected for variation in serum albumin concentration using an average correction factor. Because individual correction factors might vary, the corrected total calcium obtained could vary considerably from actual values, therefore caution needs to be taken when interpreting these calculated values (Pain et al., 1975). Other data reports that some pathology may not present an interindividual variation for correction factors, being corrected total calcium in patients with the same disease an adequate measurement of the calcium concentration in blood (Pain et al., 1980). Similarly, in cases of hypoproteinemia, low

total serum calcium concentration may not be associated with low concentrations of ionized calcium (Kanis and Yates, 1985). Thus any disorder resulting in abnormal plasma proteins may influence the amount of protein bound to calcium, resulting in changes in total calcium but without affecting the concentration of ionized calcium (Kanis and Yates, 1985). This has been proposed to be the result of the Donnan effect, whereby calcium ions are attracted electrostatically to albumin within the vascular compartment increasing total calcium without affecting the ionized fraction (Fogh-Andersen et al., 1993). When there is a difference in charges between two spaces separated by a permeable membrane, anions and cations interchange until the Donnan equilibrium is achieved. For that reason, measuring serum ionized calcium in the presence of increased albumin may overestimate the concentration of ionized calcium in the interstitial fluid, indicating that ionized calcium does not provide a true gold standard of the interstitial fluid ionized calcium status (Kanis and Yates, 1985).

There is a significant relationship between serum total calcium concentration and serum albumin. The correlation coefficients vary between species studied, being in cattle a weak correlation compared to dogs, cats and horses (Bienzle et al., 1993). There is an important correlation between ionized calcium and the concentration of serum albumin in control and hospitalized subjects (Butler et al., 1984).

Calcium Regulation

Because of the importance of calcium in different organ systems, its levels are tightly regulated, and it is the ionized calcium fraction that is susceptible to this regulation. The hormones implicated in Ca^{2+} regulation are known as calciotropic hormones, and include parathormone (PTH), vitamin D_3 and calcitonin (Fig 2-3).

Vitamin D_3

Intestinal calcium absorption can occur as passive non-saturable transport (paracellular pathway) or by active transcellular transport, both being regulated by hormones. Transcellular transport is mainly regulated by the active form of vitamin D_3 or cholecalciferol, also know as 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) or calcitriol. Two hydroxylation reactions are required for vitamin D_3 activation. The first takes place in the liver, where the 24,25-$(OH)_2D_3$ form can be stored for months, and the second hydroxylation occurs in the kidney under the control of PTH. When vitamin D_3 is activated, it promotes the absorption of calcium through the enterocytes. Activation of vitamin D_3 is induced by low blood calcium. Calcitriol also has the effect of reducing excretion of calcium through urine, and increasing the mobilization of calcium from bone to blood (Jones et al., 1998, Perez et al., 2008).

Parathormone (PTH)

Parathormone is secreted by the parathyroid chief cells of the parathyroid glands under the stimulus of low ionized blood calcium, or high phosphate levels. Parathormone acts upon bone, renal tubules and intestine to elevate the concentration of calcium when it is needed. In the bone, it activates bone resorption, releasing calcium and phosphorus from the matrix to the blood, and in the distal and collector ducts of the kidney, PTH increases reabsorption of calcium and decreases reabsorption of phosphorus. Finally, PTH promotes, in the kidney, the second hydroxylation of vitamin D to produce calcitriol, having a final effect in the intestine by increasing calcium absorption as it was described above (Guyton and Hall, 2006, Lee and Partridge, 2009). The effect of PTH over specific receptors in the intestine is currently being investigated (Gentili et al., 2003, Picotto et al., 1997).

Calcitonin

Calcitonin is a hormone that produces the opposite effects of the previous calciotropic hormones. It is secreted by the parafollicular cells of the thyroid gland and it decreases the levels of ionized calcium in blood as a response to high blood calcium. This hormone promotes a reduction in calcium mobilization from bone to blood, acting upon osteoclast activity (Renkema et al., 2008).

Calcium in the Bovine

The adult cow and hypocalcaemia

In the bovine, calcium in blood is maintained in the range of 2.1 to 2.5 mmol/L (8.5-10 mg/dL), measured as total calcium. The major problem in this animal species, in terms of calcium homeostasis, is hypocalcemia, which occurs when blood total calcium drops below 2.0 mmol/L. Hypocalcemia is considered a pathologic process of the peripartum period, mostly affecting older dairy cows. It is apparently associated with inadequate mobilization of calcium from bone at a time of rapidly increasing calcium demand during lactogenesis. Hypocalcemia in older cows could also be due to a lower number of receptors for calcitriol in the intestine, as is observed in other mammals as age increases (Horst et al., 1990).

Clinical and subclinical forms of hypocalcemia are described. The clinical form, also known as milk fever or periparturient paresis, takes place when blood total calcium is below 1.38 mmol/L (5.5 mg/dL), and it can be life threatening if not diagnosed and treated adequately. The clinical signs shown by cows with hypocalcemia were already described in 1897 by Schmidt. He described cows being excited and restless, recumbent, and finally comatose. Digestion is suspended, the cow appears tympanic, constipated, and with urine retention. Pulse is weak, respiration is fast and there is often a low body temperature (Murray et al., 2008). All these clinical signs are the result of the failure of a wide number of physiological functions due to low

ionized calcium in blood. Treatment usually consists of calcium supplementation intravenously (8 to 10 g) or orally (100 g) (Doze et al., 2008, Goff and Horst, 1993).

There are several prophylactic options to reduce the risk of hypocalcemia in cows. The most commonly used is the prepartum anionic diet, which decreases the incidence of milk fever by inducing a metabolic acidosis in the cow that increases bone calcium resorption and calcium absorption in the intestine, due to an increased response to PTH (Goff et al., 1991). Another effective preventive option is using a prepartum diet deficient in calcium (Van de Braak et al., 1987), which stimulates PTH secretion. A less common method of prevention is supplementation of the cow with vitamin D or its metabolites (calcitriol or 1α-hydroxivitamin D). Two potential difficulties encountered with this methodology include the need for precise prediction of day of parturition and the danger of producing metastatic calcification of soft tissues (Bar et al., 1985).

Subclinical hypocalcemia occurs when total calcium concentration is between 1.4 and 2.0 mmol/L. Because of the absence of clinical signs, treatment is not normally performed unless hypocalcemia is suspected due to the presence of predisposing factors (Houe et al., 2001). Clinical and subclinical hypocalcemia are associated with several peripartum conditions in the cow including uterine prolapse, displacement of the abomasum, retained fetal membranes, prolonged time to first ovulation, negative energy balance, mastitis, metritis and endometritis (Curtis et al., 1983, Goff and Horst, 1997, Massey et al., 1993, Risco et al., 1994, Risco et al., 1984, Whiteford and Sheldon, 2005).

Calcium in the calf

Blood calcium levels in the calf have been widely reported to provide reference values. In contrast to adult cows, no mention of hypocalcemic states in the calf have been reported besides iatrogenic hypocalcemia after fluid therapy in calves with diarrhea (Grove-White and Michell, 2001).

When the calf is born, calcium in blood is higher than the values obtained in adult cows (Agnes et al., 1993, Cabello and Michel, 1977, Garel and Barlet, 1976, Szenci et al., 1994), however no association between blood calcium of the dam with the levels obtained in their offspring has been observed (Szenci et al., 1994). The age at which calves develop adult-like calcium levels in blood have not been well established, with conflicting findings among studies (Agnes et al., 1993, Cabello and Michel, 1977, Dubreuil and Lapierre, 1997, Garel and Barlet, 1976, Szenci et al., 1994).

The importance of blood calcium levels to neonatal health has not been well documented. In one study, calves with signs of septicemia and high levels of tumor necrosis factor (TNF) had lower values of ionized serum calcium than those with normal levels of TNF, but the study was not designed to show an association between serum calcium concentrations nor did it determine a temporal relationship between calcium and TNF concentrations (Basoglu et al., 2004). In the study performed by Cabello and Michel (1977), plasma total calcium was measured during the first twenty days of life in dairy calves in two groups, healthy and diarrheic calves. They found a significant, almost constant, difference during all periods of the study between healthy and diarrheic calves. They also found differences in albumin concentration, in total protein and globulin (measured as the subtraction of albumin from total protein) between the two groups. This difference in globulin between groups could have confounded their results. Another study reported greater total calcium levels, but lower ionized calcium, in healthy calves compared to calves with diarrhea (Grove-White and Michell, 2001).

The Immune System

Introduction

The immune system is composed of cells and molecules, and the immunity provided by the immune system is classified as innate and adaptive. Innate immunity is the first line of defense

against any harmful insult, but is less specific than adaptive immunity. It is formed of physical and chemical barriers that control the entrance of foreign particles, as well as cells that recognize and eliminate those particles once they have entered the body. Some of these barriers are the skin and mucous membranes, antimicrobial substances and cells like macrophages, neutrophils and natural killer cells (Murphy et al., 2008).

Adaptive immunity, being more specific, needs more time to develop, and therefore is the second line of defense in the body. It takes days to develop, but it is able to eliminate many infections more efficiently than the innate immune response. This type of immunity is characterized by antigen specificity, diversity, immunologic memory and self/nonself recognition. This immunity is composed of cells (lymphocytes and antigen presenting cells) and their products (e.g. antibodies) (Goldsby et al., 2000).

These two components of the immune system need to work in cooperation with one another to provide adequate protection against microbial pathogens.

Bovine Neonatal Immune System

Ontogenesis

Ontogeny of the immune system starts early in the development of the fetus. Studies have reported the presence of immune components at different stages of fetal development. From bovine fetuses collected at slaughter, T lymphocytes were demonstrated in the thymus at three months of gestation, and these remain at a constant rate until the birth of the calf. In the spleen and peripheral blood, the quantity of T lymphocytes is greater as fetal age increases (Senogles et al., 1979).

The proportion of B lymphocytes and monocytes is less than the proportion of T lymphocytes. B lymphocytes are fairly constant throughout gestation, being in greater proportion in the thymus, while monocytes increase in number in the thymus, spleen and peripheral blood as

gestation advances. Both, T lymphocytes and monocytes are not present in peripheral blood at three months of gestation. Monocytes appeared around four months of gestation in peripheral blood, and T lymphocytes appear too on that stage increasing rapidly its number. B lymphocytes are present at three months of gestation but in low in number throughout fetal development (Senogles et al., 1979).

The distribution of T lymphocyte subsets in peripheral blood has also been investigated. Wilson et al. (1996) found that CD2, CD8 and CD4 T cells in fetuses at 8 months of gestation are in similar proportion to those found in the adult bovine. These authors also followed the dynamics of T lymphocytes in several lymphoid tissues (spleen, thymus and mesenteric lymph nodes). The authors suggest that the reduction in peripheral blood of the various T cell subsets found between fetal calves and newborn calves could be due to heavy trafficking of these cells to secondary lymphoid tissues (Wilson et al., 1996).

In the study of lymphoid tissue formation, the thymus, spleen and some lymph nodes (prescapular and prefemoral) can be identified at seventy days of gestation, while Peyer's patches and tonsils are only identified by mild infiltration of lymphocytes at 120 and 150 days of gestation, respectively. In the early stages of differentiation, the cells that are contained in lymphoid tissues are primitive lymphocytes and hematopoietic cells and after 150 days of gestation, the organs appear more organized and contain more mature lymphocytes (Ishino et al., 1991).

Immunoglobulin (Ig) containing cells are present in the early fetal stages (Ishino et al., 1991, Schultz et al., 1973). B lymphocytes could be initially detected in lymph nodes at 90 days of gestation. The M isotype of immunoglobulins is the first to appear, and at 150 days it is the prominent isotype. Isotype G can be initially detected at 150 days and increases as the fetus

grows. Finally, IgA-producing cells are found at day 180, and remain in low numbers until the end of gestation (Ishino et al., 1991).

The immune system in the calf

Besides being born with a complete immune system, the calf is not yet able to mount an effective immune response to fight infections. Like newborns from other species, calves need the protection transferred by the mothers, mainly in the form of immunoglobulins. These passively derived immunoglobulins allow the calf to fight infections in a more specific, fast and potent way than if they had to only rely on their own naïve immune system.

During gestation in primate species, protective antibodies pass through the placenta from the mother to the fetus, providing the newborn a highly effective protection against pathogens from the first days of life until they are able to generate their own protective immunity through natural infection or vaccination (de Voer et al., 2009, Gonik et al., 2005, Redd et al., 2004, Simister, 2003). However, the type of bovine placentation (syndesmochorial) prevents the transplacental transfer of maternal antibodies. It is, therefore, important in the calf, like in the piglet (Jensen et al., 2001, Leary and Lecce, 1979), to obtain an adequate transfer of maternal immunity by absorption of Ig from colostrum (Jensen et al., 2001). In addition to being a rich source of Ig, bovine colostrum contains other immune factors such as cytokines and large number of viable maternal leukocytes.

Since it was discovered that calves are agammaglobinaemic when they are born (McEwan et al., 1970) failure of passive transfer of maternal Ig to the calf via colostrum has been widely investigated. Several studies report the various risk factors associated with failure of passive transfer (Beam et al., 2009, Trotz-Williams et al., 2008), its effect upon growth, disease incidence and mortality (Donovan et al., 1998, Robison et al., 1988), as well as the importance of

assuring colostrum feeding to the calf in the first hours of life (Matte et al., 1982, Stott et al., 1979).

Bovine colostrum is the optimal source of antibody to the calf. In the scientific literature there are many feeding schedules, volumes and Ig concentrations, as well as storage options and nutraceutical formulas that provide different antibody protection levels to the calf (Godden et al., 2006, Godden et al., 2009a, Godden et al., 2009b, Godden et al., 2003, Johnson et al., 2007, Swan et al., 2007). This protection is mainly due to the content of immunoglobulins, but the effect that other immunologic components of the colostrum, like maternal leukocytes and cytokines, have recently been elucidated in the calf (Aldridge et al., 1998, Donovan et al., 2007, Hagiwara et al., 2000, Reber et al., 2008a, b, Reber et al., 2005, Reber et al., 2006, Stelwagen et al., 2009, Yamanaka et al., 2003).

Although calves lack antibodies when they are born and their T and B lymphocytes are naïve to pathogens, their monocytes and neutrophils are able to undergo phagocytosis and respiratory burst activity in the attempt to fight the infections that they have to face (Kampen et al., 2006, LaMotte and Eberhart, 1976, Menge et al., 1998). This ‘immature’ cellular immune function needs to undergo changes in leukocyte population until values in the range of adult animals are achieved. These changes related with calf age, have been reported (Ayoub and Yang, 1996, Foote et al., 2007, Kampen et al., 2006, Mohri et al., 2007, Nonnecke et al., 2003). In spite of this immaturity the ability of the calf immune system to develop a cellular immune response comparable to that seen in adults has been reported after early vaccination (Nonnecke et al., 2005).

A Closer Look at the Immune System

The immune system is as wide as it is complex. It would be impossible to present a fair literature review of all its components, functions and regulations. Therefore it is my intention to take a close look at only those parts that will have significance to the research presented here.

The neutrophil

Polymorphonuclear neutrophil leukocytes (PMN) are the first line of defense against tissue invading pathogens. Under normal physiologic conditions, they are only present in blood, but when there is an infection they are rapidly mobilized to the infection site. This characteristic is mainly provided by its multilobulated nucleus that allows the neutrophil to accommodate its shape easily between cell junctions, and as such, is the first phagocytic cell to arrive at the affected site (Paape et al., 2003).

Neutrophils originate in the bone marrow from hematopoietic stem cells that are the common precursors of the cells of both the innate and adaptive immune systems (Murphy et al., 2008). These cells, following further differentiation, become granulocytes (neutrophils, eosinophils and basophils).

Neutrophils, as other granulocytes, contain cytoplasmic granules. The cytoplasmic granules found in the bovine neutrophil are classified as primary or azurophilic, secondary or specific, and 'novel' granules (Figure 2-4) (Gennaro et al., 1983a, Paape et al., 2003). Primary granules have peroxidase activity, are round or elongated, and are present in a small number in the bovine. Specific and novel granules are peroxidase negative, and therefore have oxygen-independent antibacterial activity. Specific granules are smaller in size (0.15-0.3 μm of diameter) and more electrodense than the 'novel' granules which are paler and greater than 0.35 μm (0.4-0.5 μm) in size. Novel granules contain highly cationic proteins with antibacterial properties

(Gennaro et al., 1983b) which are released following phagocytosis but also after being stimulated with phorbo myristate acetate (PMA), similarly to the specific granules (Gennaro et al., 1983a).

Neutrophils are stimulated through membrane receptors that are triggered by specific ligands. Once activated, there is an intracellular ion flux to initiate the neutrophil response. Chemotaxis, phagocytosis, mobilization of granule content and oxidative burst are processes that the neutrophil undergoes following activation (Styrt, 1989).

When foreign microbes enter the body, they first encounter tissue macrophages at the site of entrance. These macrophages are activated by the presence of the microbes and release chemokines and cytokines, producing an inflammatory reaction with endothelial activation. Endothelial activation comprises vasodilatation, expression of adherence molecules in the endothelial cells, and increased vascular permeability. These processes lead to the recruitment of PMN to the site of infection.

To enter the site of inflammation, the PMN must first roll along the endothelial surface. This occurs when endothelial surface molecules interact with L-selectins (adhesion molecules of leukocytes) of the PMN causing deceleration of the PMN. A second group of molecules will produce a tight binding of the PMN to the endothelium. Some molecules involved in this mechanism are LFA-1 (leukocyte functional antigen 1) and CR3 (complement receptor 3), which are beta-integrins present on the surface of the PMN which interact with endothelial molecules like ICAM-1 (intracellular adhesion molecule 1) (Burg and Pillinger, 2001). The last steps in the migration of neutrophils are diapedesis through the endothelial membrane and the actual migration through the tissues along a chemotactic gradient.

Once the PMN has arrived at the infection site, it encounters the microorganism and will try to phagocytize it. Within neutrophils are lysosomes that contain enzymes and molecules that

can produce cell damage. When the neutrophil has engulfed bacteria in a phagosome, the phagosome fuses with a lysosome and its content causes destruction of the pathogen.

Some molecules present on the surface of the neutrophil, which stimulate phagocytosis, are complement receptors (CRs) and receptors for the crystalizable fraction of immunoglobulins (FcR). Therefore, bacteria opsonized by complement factors or aggregated by Ig are phagocytized following activation of the neutrophil.

Reactive oxygen species (ROS) production by neutrophils is called the respiratory burst. This is initiated by the reduction of molecular oxygen (O_2) to superoxide anion (O_2) by NADPH-oxidase. NADPH is the donor of an electron to the oxygen to produce the reaction:

$$NADPH + 2\ O_2 \rightarrow 2\ O_2^- + NADP^+ + H^+$$

Different subunits of NADPH- oxidase need to be assembled for it to be active. NADPH-oxidase requires phosphorylation for its activation (Babior, 1999, Waki et al., 2006). Phosphorylation of one of the subunits, $p47^{PHOX}$, is regulated by several kinases, the most important of which is protein kinase C (PKC) (Park et al., 1997, Tauber, 1987, Waki et al., 2006, Wolfson et al., 1985, Yamamori et al., 2000). Protein kinase C can be activated by endogenous diacylglycerol (DAG) or by exogenous phorbol-sters like PMA (that produce its action by mimicking the mechanism of the DAG). The PKC in non-stimulated neutrophils is found in the cytosol of the cell, and following stimulation (PMA, opsonized zymosan and heat-aggregated IgG) migrates to the cytoplasmic membrane where NADPH-oxidase activation and superoxide anion production occurs. Protein kinase C is also involved in neutrophil phagocytosis (Waki et al., 2006).

In the sheep, superoxide anion is released by neutrophils when these are stimulated with PMA (phorbol myristate acetate), but not with PAF (platelet activating factor), TNFα, LPS or

fMLP (N-formyl-methionyl-leucyl-phenylalanine), and the reaction increases when neutrophils are incubated with PMA plus PAF (Tung et al., 2009). In the bovine, opsonized zymosan (OPZ) stimulates the production of superoxide anion but needs the presence of complement receptor 3 (CR3) on the neutrophil surface for a proper response (Higuchi and Nagahata, 1998, Nagahata et al., 2007). To induce activation and superoxide production of neutrophils through the Fc receptor, heat aggregated IgG (H-agg.IgG) has been successfully used (Higuchi and Nagahata, 1998). Differences have been found between cows and calves under 5 days of age in the production of superoxide by neutrophils stimulated by several mechanisms. When H-agg.IgG and PMA were used, adult cows produced a significant increased production of superoxide anion but when OPZ was used, calves shown an increased production of O_2^- compared to adult cows (Higuchi and Nagahata, 1998).

Cytokines

Cytokines are soluble proteins synthesized and released by cells following stimulation. Cytokines released after cell activation will act upon their same or other cells, producing either a stimulatory or inhibitory effect. Because of the complexity of cytokine biological mechanisms, the idea of 'cytokine network' was suggested and network analysis was used as an aid to understand its complex interactions (Tieri et al., 2005).

Cytokines are involved in the early innate inflammatory response initiated by any foreign body. As I have described before, tissue macrophages are the first cells to recognize the entrance of pathogens. This recognition is mediated by receptors on the macrophage surface that recognize, in a non-selective way, molecules present on the surface of the pathogen. These receptors are the toll-like receptors (TLR) and form part of a family called pattern recognition receptors (PRR). Eleven TLR have been identified in mammals (Takeda and Akira, 2005) and in the bovine ten TLR have been characterized (McGuire et al., 2006). When TLRs bind to non-

specific molecules present on the microorganism surface called pathogen associated molecular patterns (PAMPs), gene transcription is initiated, producing the synthesis of cytokines and initiating the immune response cascade. After the entrance of an invading pathogen, there is an initiation of an inflammatory response lead by proinflammatory cytokines. These proinflammatory cytokines need to be controlled by anti-inflammatory cytokines, or otherwise, massive tissue destruction and other negative consequences for the host would take place. Some pro-inflammatory cytokines are IL-1, IL-6, IL-12, TNF-alpha and IFN-gamma and examples of anti-inflammatory cytokines are IL-4, IL-10 and IL-13.

Interferon gamma (IFN-gamma)

Interferon gamma is an important cytokine that modulates the immune response. Its activity has been associated with T helper lymphocytes type 1 (Th1) (Figure 2-5) and its production has an effect on macrophage stimulation, in class switching of B lymphocytes and in stimulating the production of Th1 over Th2 cells. Under pathogen stimulation, natural killer cells (NK) produce IFN-gamma, priming monocytes to produce tumor necrosis factor alpha (TNF-alpha) and interleukin 12 (IL-12). Later in the response, more IFN-gamma is produced by activated T lymphocytes (Billiau and Matthys, 2009). In the calf, peripheral blood mononuclear cells have been able to produce IFN-gamma in response to stimulation with *Mycobacterium bovis* derived purified protein derivative (PPDb) (Foote et al., 2007) and the production by NK cells is similar or greater, depending on the presence of other cytokines, in the calf under one week of age, compared to older calves (Elhmouzi-Younes et al., 2009).

Tumor necrosis factor-alpha (TNF-alpha)

Tumor necrosis factor-alpha is a cytokine produced by macrophages in response to the activation of TLR by bacterial compounds. Secretion of TNF-alpha is accompanied by the

production of some other proinflammatory cytokines (IL-12 and IL-6). Production of TNF-alpha by bovine macrophage is increased when macrophages are under the stimulus of IFN-gamma (Werling et al., 2004). When neutrophils are stimulated with bacterial peptides in the presence of TNF-alpha, the oxidative burst response is primed, with an increased production of H_2O_2 (Gougerot-Podicalo et al., 1996). TNF-alpha plays a potentially damaging role in animals suffering from bovine respiratory disease, inducing the activation and degranulation of neutrophils (Wessely-Szponder, 2008). Increased levels of TNF-alpha have being associated with lung tissue damage in both human and animals due to its correlation with ROS production by stimulated neutrophils (Gougerot-Podicalo et al., 1996, Yoo et al., 1995).

Calcium and The Immune System

Mechanism of calcium in immune cells

Calcium (Ca^{2+}) is an important regulatory signal in the activation of cells of the immune system (Baine et al., 2009, Brechard et al., 2008, Brechard and Tschirhart, 2008, Feske, 2007). This activation consists of cell differentiation, gene transcription and effector functions.

The mechanism by which Ca^{2+} acts as a second messenger in the activation of immunologic cells has been mostly investigated in T lymphocytes. The mechanism is presented in Figure 2-6 and will be discussed below.

When the T-cell is presented an antigen through its T cell receptor (TCR), the stimulatory response initiates activation of tyrosine kinases which after some complex processes will activate phospholipase C (PLC). Phospholipase C catalyzes the hydrolysis of membrane phospholipids, resulting in the formation of inositol triphosphate ($InsP_3$) and diacylglycerol (DAG). Inositol triphosphate binds to $InsP_3$ receptors on the surface of the endoplasmic reticulum (ER), leading to the release of Ca^{2+} from the ER to the cell cytosol. This leads to a short-lived and moderate increase in intracellular Ca^{2+} concentration. More importantly, however, the decrease in Ca^{2+}

concentration in the ER activates the opening of calcium release activated calcium (CRAC) channels in the plasma membrane that allows extracellular Ca^{2+} to enter into the cell. These CRAC channels remain open for the time that the ER Ca^{2+} levels are low. The levels of intracellular Ca^{2+} can remain elevated for minutes to hours (and potentially, for days) (Quintana et al., 2005).

Once intracellular Ca^{2+} increases as a result of ER transfer and open CRAC channels, one of two responses can occur. In the rapid response there is no gene transcription. Examples include the Ca^{2+}-dependent degranulation of allergen exposed mast cells (within minutes of the activation) or the target cell killing by cytolytic T cells (within a few hours). In the long term response, transcription is initiated via the following pathways. In the presence of high intracellular $Ca^{2+,}$ the calcium dependent enzyme, calcineurin, is activated, which leads to phosphorylation of the nuclear factor of activated T cells (NFAT) that enters into the nucleus to begin the transcription. On the other hand, elevated levels of DAG, in the presence of high intracellular Ca^{2+} will activate other transcription factors. These transcription factors will then lead to the transcription of genes that regulate cell proliferation and differentiation; 75% of these genes show dependence on the entrance of Ca^{2+} through the CRAC channels to be activated (Quintana et al., 2005).

A reduction of intracellular Ca^{2+} levels, which can occur when serum Ca^{2+} is low, can reduce T-cell activation and proliferation (Quintana et al., 2005). Also, the lack of Ca^{2+} mediated signals has been reported to impair IL-2 production and T-cell production *in vitro* and to produce a defective T-cell mediated immune response *in vivo* (Feske et al, 2007).

Action of calcium in neutrophils

Neutrophil activation results in an influx of Ca^{2+} into the neutrophil that is dependent on the concentration of extracellular Ca^{2+}. External Ca^{2+} is also needed for the generation of the

oxidative burst (Cudd et al., 1999, Ortiz-Carranza and Czuprynski, 1992). If neutrophils are activated in the presence of verapamil, which is an inhibitor of Ca^{2+} channels, the response of the neutrophils to a stimuli decreases significantly (Yu and Czuprynski, 1996).

This Ca^{2+} dependent activation of neutrophils depends on the type of stimulus applied. Some neutrophil receptors are Ca^{2+} dependent, while others are able to produce activation even in the absence of Ca^{2+}. For example, neutrophil activation through CR3 and Fc receptors, which occurs with OPZ and H-AggIgG, depend on Ca^{2+} (Yu and Czuprynski, 1996), while in neutrophils stimulated with PMA that dependence is not found (Leino and Paape, 1996). This is probably due to the activation mechanism of PMA which has DAG-like activity.

Another possible role of Ca^{2+} in immune function has to do with control of intracellular alkalinization. Neutrophil functions, such as cell migration, microbiocidal behavior, granule exocytosis and intracellular ROS generation, are sensitive to intracellular pH fluctuations. It appears that there is an initial acidification following neutrophil stimulation, followed by a more sustained alkalinization that is dependent on Ca^{2+} influx. This alkalinization is inhibited in a Ca^{2+} free medium, when Ca^{2+} is chelated, or when store-operated calcium entry (SOCE) channels are inhibited (Sandoval et al., 2007).

Action of calcium in cytokine production

Degranulation and release of cytokines is another mechanism in which Ca^{2+} is involved in the immune response. Production of interleukine-4 (IL-4) and TNF-alpha by basophils can be inhibited by substances that inhibit the increase of intracellular Ca^{2+} (Wang et al., 2007). Inhibition of cytokine production by T cells (IL-2, TNF-alpha and IL-17) occurs when cells are incubated in media with CRAC inhibitors (Di Sabatino et al., 2009).

Besides the effect of Ca^{2+} on cytokine secretion, a reduction of cytokine gene expression has been reported when SOCE channels are inhibited. This inhibition produced a decrease in IL-

2 secretion and lower IL-2 and NFAT gene expression after cell activation (Ishikawa et al., 2003).

Calcium and the Immune System in the Bovine

In the cow the relationship between serum Ca^{2+} and immune response is just now being elucidated. Some studies have reported a relationship between Ca^{2+} and resistance to infection (Bagnall et al., 2009).

Kimura et al. (2006) showed that cows with clinical milk fever (clinical hypocalcemia) had lower Ca^{2+} in the ER of peripheral blood mononuclear cells (PBMCs) and lower calcium influx into PBMCs after being stimulated, and that treatment with intravenous Ca^{2+} improved the influx of Ca^{2+} into PBMCs. This could be one reason why cows with clinical hypocalcemia are at increased risk of post-parturient infections.

Some genetic defects in neutrophil receptors have been identified. The CC genotype of the CXCR 1 receptor, which can be activated by IL-8, is one of those defective receptors. These receptors can still be activated by IL-8, but the normal response is not produced, resulting in reduced influx of Ca^{2+} into the neutrophil. This results in impaired neutrophil function, and is associated with an increase in clinical mastitis (Rambeaud and Pighetti, 2005, 2007, Youngerman et al., 2004).

Summary

Neonatal calves are at a great risk of suffering infectious diseases, due to the lack of a mature immune system and to the presence of various pathogenic microbes in the environment that surrounds the calf.

The calcium molecule has been identified as an important cell messenger involved in an adequate immune response, and the effect that hypocalcemia in the cow has over immune cells'

function is beginning to be studied, showing some interesting relationships between blood calcium and cell function.

Therefore, it seems worthwhile to investigate the role that serum calcium plays in disease resistance in the calf and this knowledge may play an important role in prevention and/or treatment of calfhood diseases.

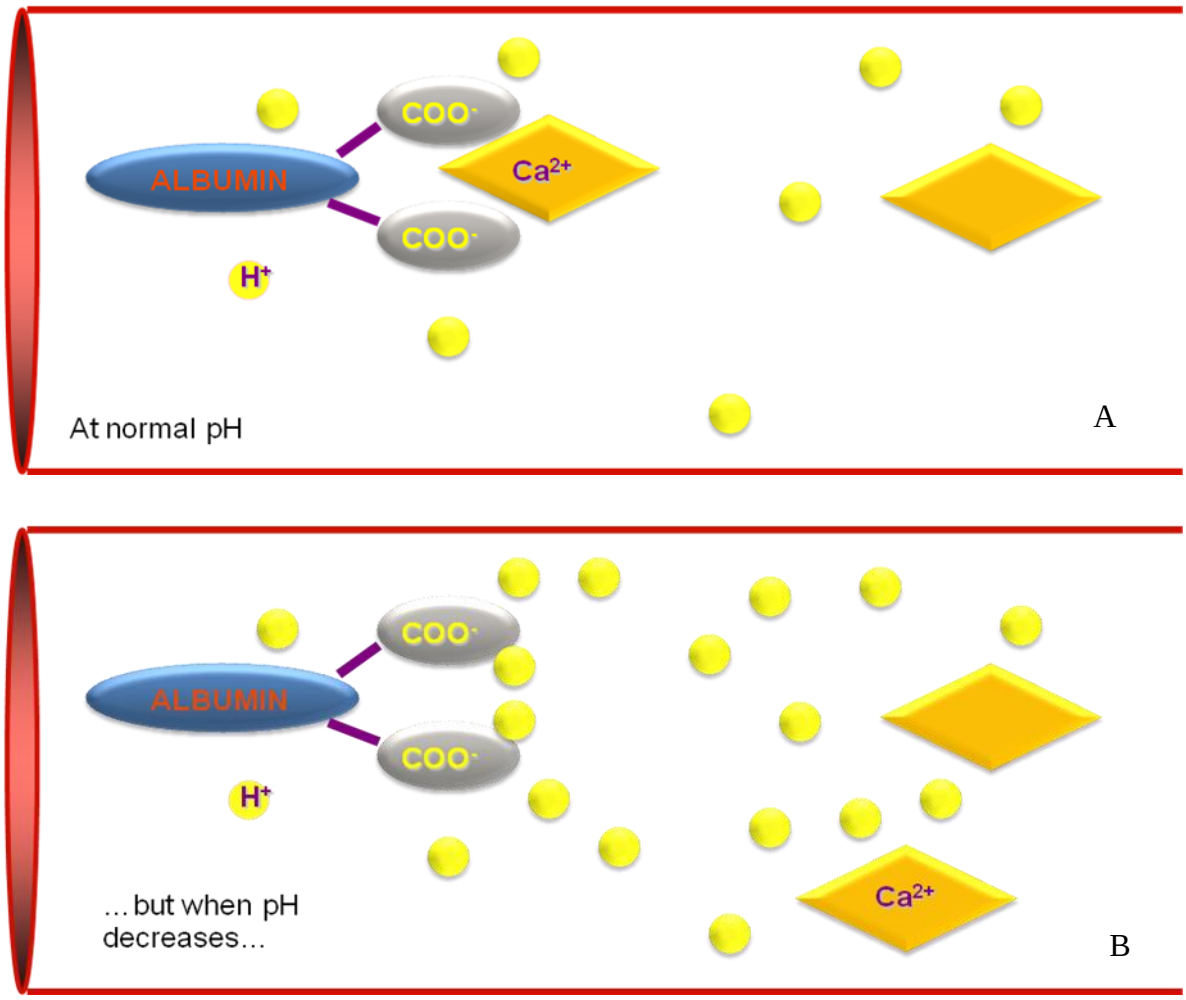

Figure 2-1. Effect of acidosis on the dissociation of the Ca^{2+} molecule from albumin. A) Under physiologic pH conditions, a fraction of Ca^{2+} is bound to albumin. B) When pH decreases in acidosis the increased number of protons in blood will displace the molecules of Ca^{2+} away from the albumin, producing an increase in Ca^{2+} in blood.

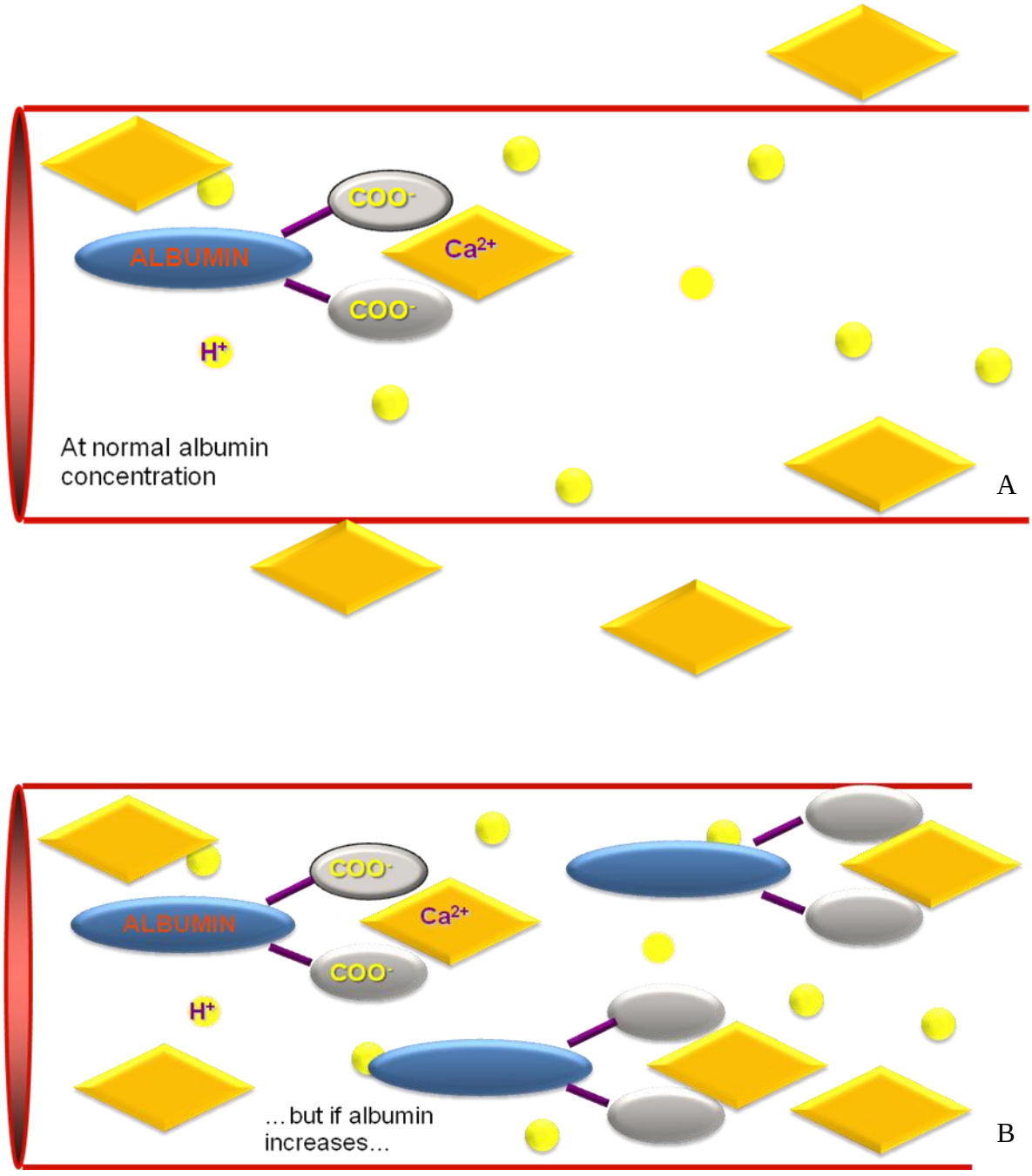

Figure 2-2. Effect of the increased blood albumin over serum total calcium. A) Under physiologic conditions, a fraction of Ca^{2+} is bound to albumin. B) When albumin increases in blood the new molecules of albumin will bind to the molecules of Ca^{2+} present in blood. To maintain the electrostatic equilibrium between the extravascular and vascular space, Ca^{2+} molecules will enter into the blood stream from the extravascular space producing an increase in total calcium.

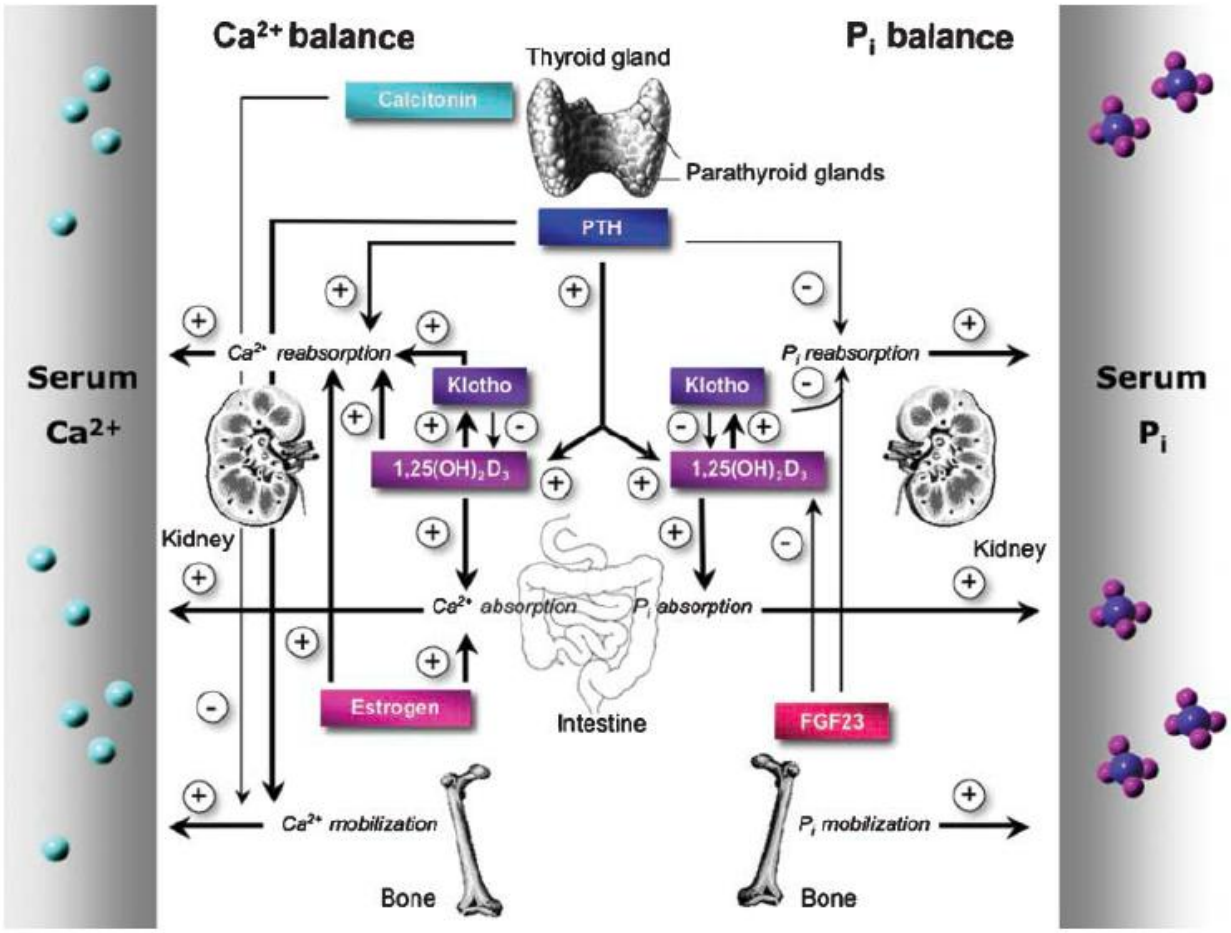

Figure 2-3. Hormonal regulation of the calcium molecule. Renkema, K. Y., R. T. Alexander, et al. (2008). "Calcium and phosphate homeostasis: concerted interplay of new regulators." Ann Med 40(2): 82-91.

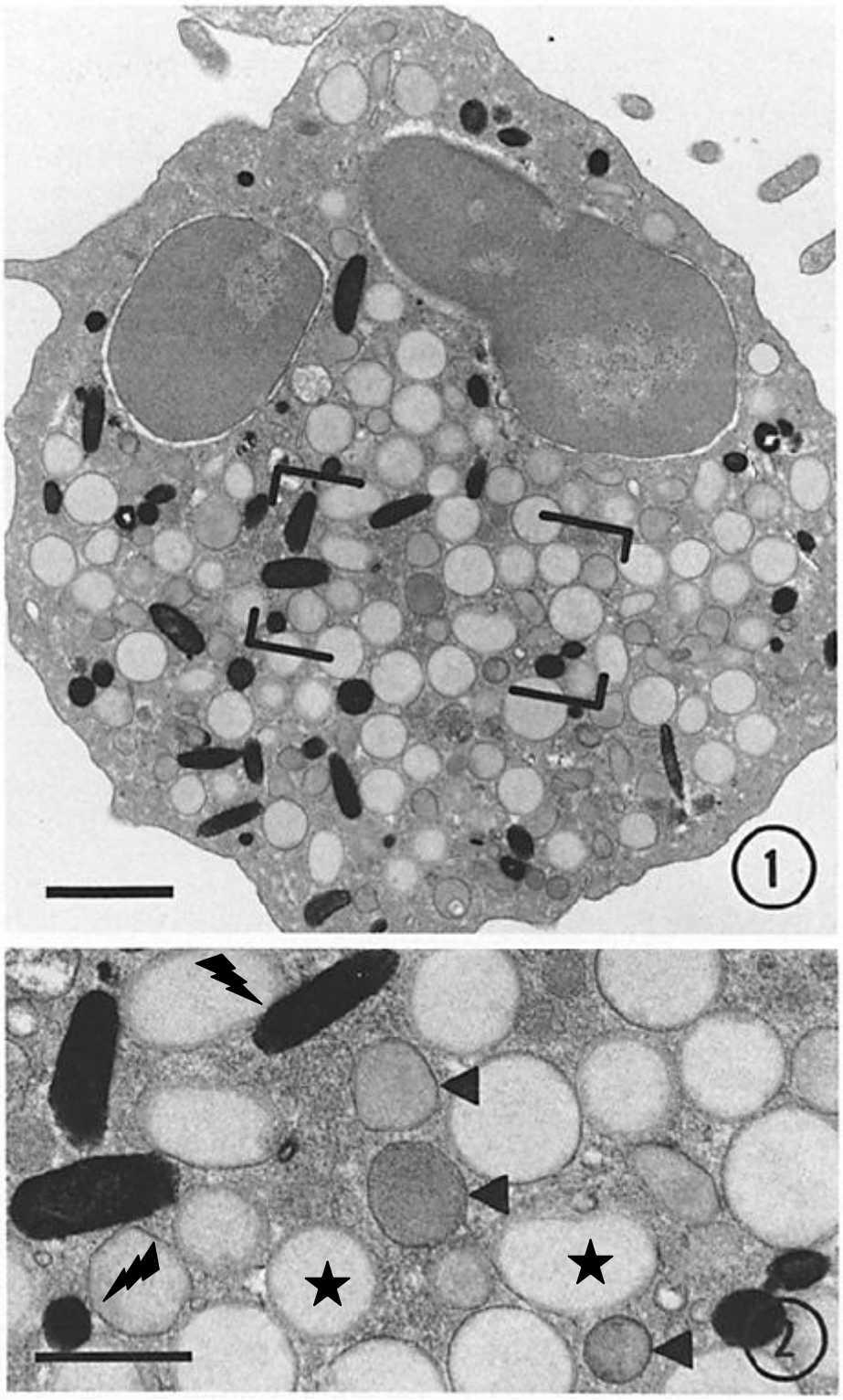

Figure 2-4. Neutrophil from bovine blood. Azurophilic granules (lightning bolt), specific granules (star) and novel granules (triangle). Gennaro, R., B. Dewald, et al. (1983). "A novel type of cytoplasmic granule in bovine neutrophils." J Cell Biol 96(6): 1651-61.

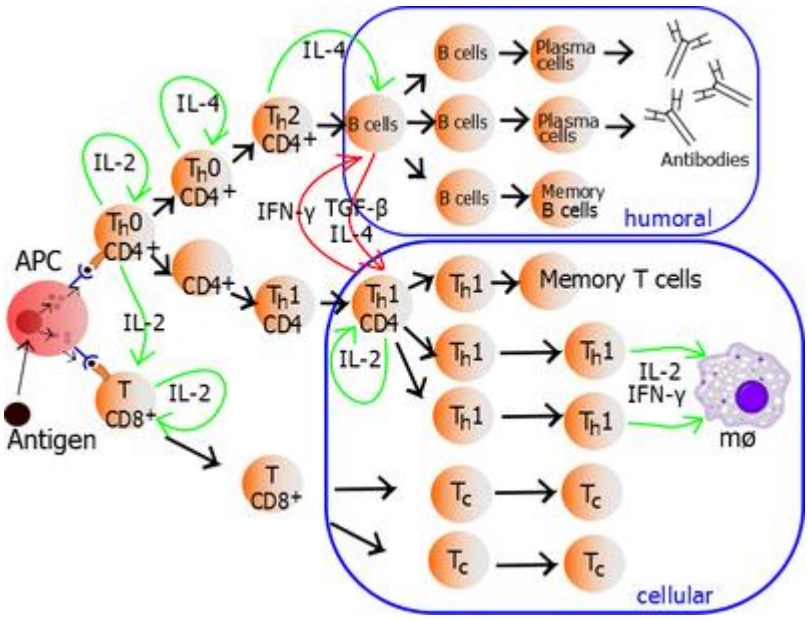

Figure 2-5. Th1/Th2 model of immune response after activation following antigen presentation by antigen presenting cells. Cytokines involved in the process. http://en.wikipedia.org/wiki/T_helper_cell

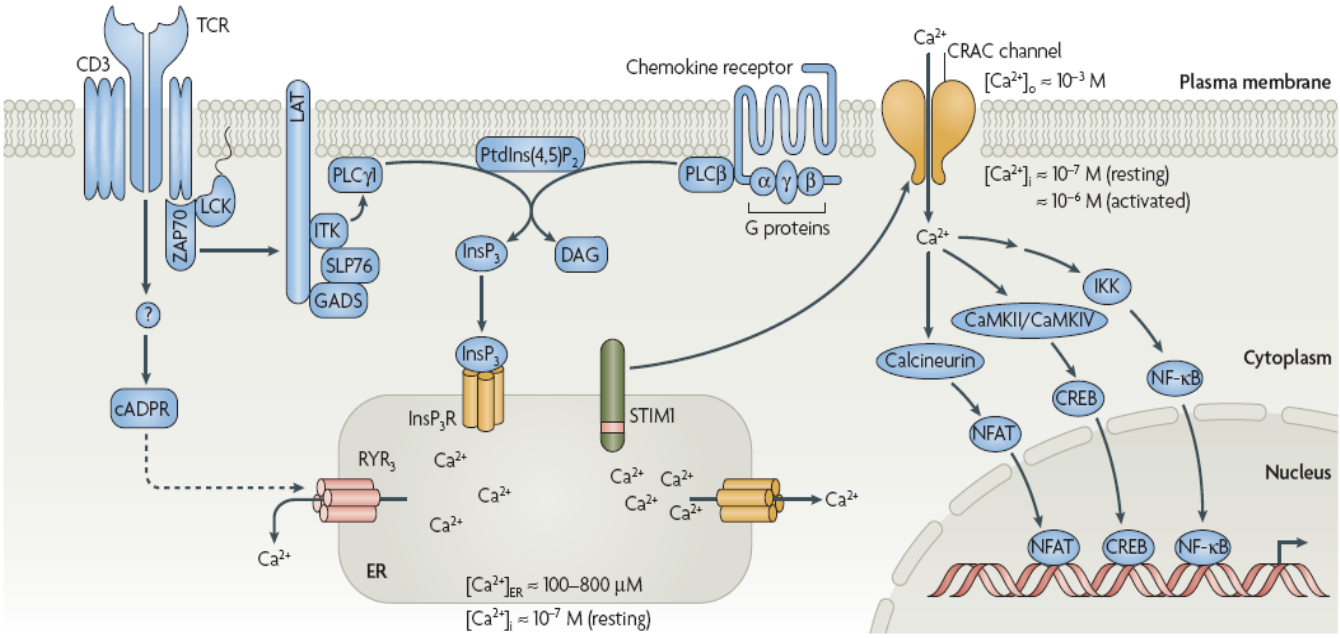

Figure 2-6. Calcium dependent activation of T lymphocytes after being stimulated through their cell receptor (TCR). Feske, S. (2007). "Calcium signalling in lymphocyte activation and disease." Nat Rev Immunol 7(9): 690-702.

CHAPTER 3
IONIZED CALCIUM VS TOTAL CALCIUM

Introduction

Historically, the concentration of calcium in blood has been investigated to obtain reference values in both healthy and diseased people yet the methods for measurement have remained controversial (Wills and Lewin, 1971). Because of the physiochemical properties inherent in elemental calcium, the laboratory measures are variable and depend on sample processing, analytic methods, and physiologic state of patients. Thus the calcium levels reported may not accurately reflect the bioactive calcium in the patient (Berry et al., 1973, Kanis and Yates, 1985, Kogika et al., 2006, McLean, 1934).

Several laboratory methods have been proposed to address these difficulties, resulting in formulae and correction factors that do not always fit as well as expected (Jain et al., 2008, Pfitzenmeyer et al., 2007). Therefore, the debate about which method is more accurate remains unresolved. In particular, the question remains if measuring ionized calcium improves the diagnosis of hyper/hypocalcemia in some diseases (Riancho et al., 1991).

Within the veterinary literature, there was no correlation between ionized calcium and total calcium when measured in dogs with blastomycosis (Crews et al., 2007). This population of dogs had an 81.6% incidence of hypoalbuminemia. In another study (Schenck and Chew, 2005), the correlation between total and ionized calcium was 0.73 in dogs with chronic renal failure and 0.87 in dogs with conditions other than chronic renal failure. In dairy calves low correlations between serum total calcium and ionized calcium, total calcium and albumin, and ionized calcium and blood pH have been reported. Some differences in correlation has been shown according to age, with stronger correlations at 2 and 3 months of age between total calcium and albumin (Agnes et al., 1993).

The objective of the study presented in this chapter is to determine the correlation between ionized calcium and total calcium in the newborn dairy calf, and how possible changes in pH and albumin in calves with diarrhea could affect their levels of serum total calcium. The data in this study will help to determine if the presence or absence of diarrhea could be an important factor in the use of serum total calcium in the main study objective of the thesis project.

Materials and Methods

A case-control study was designed for this experiment.

Animals

A total of 20 Holstein dairy calves, both males and females, between 4 and 11 days of age were enrolled in this study. Cases were selected randomly from those calves that were observed with signs of clinical diarrhea, without clinical dehydration or depression (Walker et al., 1998). Ten calves were included in this group, 6 males and 4 females, and all were under supportive treatment for diarrhea for one to four days. The supportive therapy consisted in oral electrolytes once a day at midday. Controls were randomly selected from those calves that did not shown any signs of diarrhea (n=10), 6 males and 4 females. Selection of controls and cases was done with the aim to minimize age differences between groups.

Calves belonged to the same farm. Females were housed in individual hutches with rubber slat flooring and with close contact between each other, while males were housed individually on dirt and with approximately 0.5 m of separation between hutches. Sanitation practices were daily flushing of the floor with water in the female housing system and relocation of the hutches onto a clean area when needed in the male housing system. Feeding and health procedures were similar for both males and females.

Samples

Serum and plasma samples were taken to measure iCa and tCa concentration. Samples were taken early in the morning. Calves were bled via jugular venipuncture using a 10 cc blood collection tube without additive and another 10 cc blood collection tube with lithium heparin (BD Vacutainer®). The samples were stored at 4 ºC until further processing. Within two hours of collection, the samples without anticoagulant were centrifuged at 1800 rpm for 15 minutes, serum collected and stored at 4 ºC, and delivered to the College of Veterinary Medicine, University of Florida to measure total calcium (tCa) and albumin using a chemistry analyzer (Hitachi 912, Roche Diagnostics©). To obtain a quantitative determination of total calcium (tCa) present in serum, the reagent (Phosphonazo III) Calcium L3K® Assay (Diagnostic Chemicals Ltd.) was used following the manufacturer's protocol. The principle of the assay is that the Phosphonazo III will react with calcium forming a complex of blue-purple color. The color has a maximum absorbance of 600 nm and the color-change is proportional to the sample calcium concentration. Results are expressed as mg/dL. These were converted to mmol/L using the formula:

tCa (mmol/L) = tCa (mg/dL) x 0.25

The assay used for the determination of albumin is based in the Bromocresol green (BCG) reaction, which forms a complex with albumin that has a maximum absorbance at 630 nm; the absorbance of the sample is proportional to its albumin concentration. The kit used for this purpose was the Albumin Assay (Diagnostic Chemicals Ltd.); the results are reported as g/dL.

Within thirty minutes of collection, samples with lithium heparin were analyzed using a portable i-STAT® machine (© Abbott Point of Care Inc., Princeton, USA) with CG8+ cartridges. The measures of interest that we obtained were ionized calcium (iCa) expressed in

mmol/L and pH. This one done always by the same researcher and under similar conditions to minimize inter-cartridge variation.

Statistical Analysis

Means from each group were compared using the Mann-Whitney test for non-parametric data, due to our small sample size. Linear correlation was investigated using Spearman's correlation test between variables. Finally a linear model was constructed to explain iCa as a function of the other variables. Initially, univariate analysis was performed. Variables with p-value ≤ 0.20 were used in a multivariable analysis with backward elimination. Variables retained in the model were those with p-value of ≤ 0.05.

PROC MEANS, PROC UNIVARIATE, PROC CORR and PROC REG procedures of SAS (SAS 9.2, SAS Institute Inc.) were used and statistical significance was stated at a p-value of less than 0.05.

Results

Descriptive statistics of blood variables from all 20 calves are presented in Table 3-1. The mean age of calves with diarrhea was significantly higher (9.2 vs 5.2) than calves without diarrhea. Total calcium and albumin were significantly ($p = 0.051$ and $p = 0.005$, respectively) different between groups. Total calcium was higher in calves without diarrhea (2.80 mmol/L vs 2.66 mmol/L) and albumin was higher in calves with diarrhea (2.90 g/dL vs 2.58 g/dL). No significant differences were found in iCa and in iCa to tCa ratio, but pH was significantly ($p = 0.051$) lower in calves with diarrhea compared to calves without diarrhea, with a difference of 0.04 units (Table 3-2).

Significant correlations were found between tCa and iCa ($p = 0.003$). Total calcium was also correlated with age and negatively correlated with the presence of diarrhea. Ionized to total

calcium ratio showed no correlation with tCa or iCa. Other correlations investigated are presented in Table 3-3.

Two models were created, one to predict tCa and another to predict iCa from the variables collected. For the first model the best fit contained iCa, pH, albumin and age. The second model fitted tCa, pH and albumin. The models are:

Total calcium (mmol/L) = -12.36 + 1.52 * iCa (mmol/L) + 1.67 * pH + 0.33 * Albumin (g/dL) – 0.03 * Age (days)

Ionized calcium (mmol/L) = 8.95 + 0.40 * tCa (mmol/L) - 1.13 * pH – 0.13 * Albumin (g/dL)

The results of the multivariate analysis and the p-values are presented in Table 3-4.

Discussion

The age between the two studied groups of calves was different, due to the age in which diarrhea develops in calves in the study farm. Infectious diarrhea normally occurs in the young calf, within the three first weeks of age. *E. coli* K-99 infection is more commonly reported within the first two days of life, and afterwards other pathogens are the cause of diarrhea (De Rycke et al., 1986, Foster and Smith, 2009, Holland, 1990). It is likely that as result of a good vaccination protocol and management of the calving area in the farm where the study was conducted, that the presence of diarrhea caused by *E. coli* was non-existent, explaining why the group of calves without diarrhea was younger.

The differences found in serum tCa between groups are likely to be due to age. Highest values of tCa have been reported at birth and then there is a decrease in different magnitude until it achieve adult cow values (Agnes et al., 1993, Cabello and Michel, 1977, Mohri et al., 2007). Another difference found between groups was pH, and this could have had an effect on the

ionized calcium in the sample due to a possible pH-related change in the binding affinity of the molecule of calcium to albumin.

The main impact of pH on blood calcium is that the increase of protons in blood that acidosis produces reduces the dissociation of the protons from the carboxyl group of the albumin, leaving no space for the attachment of calcium molecules to albumin (McLean, 1934). This results in increased iCa with no change in total calcium. In 1971, Wills and Lewin reported that calcium-binding affinity of the plasma proteins, determined by calcium proteinate dissociation constant (Kcaprot), under physiological temperature and pH conditions, did not vary significantly between normal human subjects (pH=7.33±0.03) and patients with hypercalcaemia (pH=7.35±0.08) and renal (pH=7.29±0.05) and non-renal (pH=7.37±0.05) hypocalcaemia. They concluded that the concentrations of ultrafiltrable, ionized and protein-bound calcium concentrations could probably be predicted from the total plasma calcium concentration unless there are marked changes in total plasma protein concentration. In this study, a difference in pH of 0.04 units, from pH 7.38 to pH 7.42, between calves with and without diarrhea was detected. This difference is within the range of pH variation that Wills and Lewin (1971) reported, although these study calves presented higher pH than the human patients in the former study. In calves with experimentally induced diarrhea, venous pH values were reduced from 7.36 to 7.31, and at low pH, clinical signs such as depression and dehydration (eyeball recession and skin tent) were markedly increased (Walker et al., 1998). Clinical depression and dehydration were not observed in the diarrheic calves sampled in the current study. Therefore, I could be confident that pH in calves with diarrhea, but no clinical depression or dehydration, will not impact significantly in the ionized calcium concentrations.

The action of serum albumin on calcium could be explained as opposite to the effect of pH. Albumin in plasma balances the intravascular hydrostatic pressure, and when albumin is increased, the molecules of ionic calcium will bind to the 'new' molecules of albumin, therefore decreasing the amount of ionic calcium in blood. Due to the Donnan effect, the permeability of capillary walls will allow ionized calcium to enter from the extravascular space into the blood stream, until the Donnan equilibrium between the extravascular and intravascular spaces is achieved. Donnan equilibrium is achieved when two virtual compartment that are separated by a permeable membrane present the same net electrostatic charge. This can be achieved following anion and cation diffusion through the membrane. The consequence is that serum total protein has increased but iCa is unchanged (Fogh-Andersen et al., 1993).

We found a higher albumin concentration in calves with diarrhea than in calves without diarrhea, which could be explained as an effect of dehydration or an age-related change. Walker et al. (1998) described an increase in serum albumin of 0.8 g/dL in calves with induced diarrhea, when signs of severe dehydration were present. In the present study, the difference was only of 0.3 g/dL, but a smaller difference of 0.4 g/dL between normal and severely dehydrated calves under ten days of age with diarrhea have been reported (Thornton et al., 1972). Changes in albumin during the first months of life in calves have also been reported. Serum albumin increases with age, with the change more pronounced during the first twenty to forty days of life, but not consistent with the findings in serum albumin presented in Chapter 4 of this thesis that reports changes with age but not an uniform increase. The difference with age in serum albumin in previous studies was from approximately 2.55 g/dL at 6 days of age to 2.80 g/dL on day 9. On day 14 of age values reported are around 3.2 to 3.3 g/dL (Knowles et al., 2000, Mohri et al., 2007, Nussbaum et al., 2002). If albumin would have had an effect in tCa, the tCa obtained in

these calves would have increased with age instead of decreased, probably being the effect of albumin on tCa of low magnitude compared to changes presented with age. Therefore the effect of albumin on tCa should be interpreted with care when comparing calves of different ages, with or without diarrhea. The comparison of serum tCa in those calves could only be reliable if there is no difference in albumin between calves with and without diarrhea for each age group.

In contrast to other studies in calves (Agnes et al., 1993) the correlation between iCa and tCa in this sample population was strong. This allowed modeling an equation to express tCa as a function of iCa, albumin, pH and age. What is more important for the next study of this thesis, another equation was developed to express iCa as a function of tCa, albumin and pH.

Therefore, by selecting only calves without diarrhea, dehydration and clinical depression (excluding animals with treatment for diarrhea) the variability in pH can be controlled, and by selecting calves of the same age we can assure no difference in serum albumin concentration, then the variation in iCa would largely be explained by the variation in tCa. This is therefore the justification for the use of tCa as an adequate method to explain the biologically active iCa concentrations in the calf in Chapter 4 and Chapter 5 of this thesis.

In the present study the iCa:tCa ratio did not change between study groups. In case of an increased albumin in calves with diarrhea due to dehydration, one would expect to see an increase in serum tCa fraction but not in the iCa fraction, resulting in a lower ratio in calves with diarrhea compared with nondiarrheic calves. In the case of pH, if the calf has acidosis, an increase in ionized calcium can occur, but not in total calcium, producing an increase of this ratio. Neither of these situations occurred in my study. In looking at the correlations, the iCa:tCa ratio was negatively, highly correlated with pH but not with albumin, indicating that if any of the

cases described above would happen in calves with diarrhea, it would be more likely result in an increase of the ratio as a result of low pH.

Conclusion

Serum total calcium results need to be interpreted carefully in dairy calves. This population of animals is at risk of having diarrhea, which can induce acidosis and hyperalbuminemia if the calf is severely dehydrated, leading to a more complicated interpretation of the laboratory results. Physiological changes in the concentration of albumin also occur with age in calves, making it even more difficult to interpret tCa levels. Therefore, ionized calcium would be the preferred method of determining calcium status in sick calves.

A significant equation of iCa as a function of tCa, albumin and pH was modeled, allowing tCa variation to explain iCa variation when the other significant variables of the model (pH and albumin) were controlled. As a result, for the purpose of the next study, serum tCa should be a reliable measure to make comparisons between calves, as we will compare animals of the same age and we will be closely monitoring calves for presence of diarrhea, removing them from sample selection if calves require therapy for the diarrhea, depression or dehydration.

Table 3-1. Descriptive values obtained from blood analysis of Holstein dairy calves with and without diarrhea.

Variable	N	Mean	Minimum	Maximum	Std Error
iCa (mmol/L)	20	1.33	1.17	1.46	0.02
pH	20	7.40	7.31	7.50	0.01
tCa (mmol/L)	20	2.73	2.53	2.95	0.03
Albumin (g/dL)	20	2.74	2.40	3.30	0.05
iCa/tCa	20	0.49	0.45	0.54	0.01

iCa = ionized calcium; tCa = total calcium; iCa/tCa = ionized to total calcium ratio.

Table 3-2. Comparison of mean values and SE and the two sided p-value of the serum analysis between calves with diarrhea and calves without diarrhea.

Group	No diarrhea		Diarrhea		
Variable	Mean	Std Error	Mean	Std Error	p-value
iCa (mmol/L)	1.35	0.02	1.31	0.03	0.299
pH	7.42	0.01	7.38	0.02	0.051
tCa (mmol/L)	2.80	0.04	2.66	0.04	0.051
Albumin (g/dL)	2.58	0.03	2.90	0.07	0.005
iCa/tCa	0.48	0.01	0.49	0.01	0.271
Age (days)	5.2	0.33	9.2	0.36	0.001

iCa = ionized calcium; tCa = total calcium; iCa/tCa = ionized to total calcium ratio; Age = age at sampling

Table 3-3. Spearman's correlation coefficient (r) and p-value between serum analytes in 20 calves.

	Statistic	iCa	pH	tCa	Albumin	Age	Diarrhea
pH	r	-0.272	1				
	p	0.245					
tCa	r	0.622	0.243	1			
	p	0.003	0.302				
Albumin	r	-0.137	-0.626	-0.229	1		
	p	0.565	0.003	0.332			
Age	r	-0.277	-0.471	-0.573	0.675	1	
	p	0.237	0.036	0.008	0.001		
Diarrhea	r	-0.245	-0.477	-0.479	0.728	0.876	1
	p	0.297	0.033	0.033	0.000	<0.001	
iCa/tCa	r	0.421	-0.707	-0.334	0.162	0.363	0.260
	p	0.065	0.001	0.150	0.496	0.115	0.268

iCa = ionized calcium; tCa = total calcium; Age = age at sampling; Diarrhea = diarrhea (yes/no); iCa/tCa = ionized to total calcium ratio.

Table 3-4. Multivariable analysis of the effect of select variables on total calcium (Model 1) and ionized calcium (Model 2). Parameter estimates and p-values.

Variable	Estimate	SE	t value	Pr > |t|
Model 1				
Intercept	-12.36	3.62	-3.42	0.004
iCa (mmol/L)	1.52	0.24	6.35	<0.001
pH	1.67	0.45	3.75	0.002
Albumin (g/dL)	0.33	0.09	3.64	0.002
Age (days)	-0.03	0.01	-3.13	0.007
Model 2				
Intercept	8.95	1.67	5.36	<0.001
pH	-1.13	0.22	-5.18	<0.001
Albumin (g/dL)	-0.13	0.05	-2.81	0.013
tCa (mmol/L)	0.40	0.06	6.34	<0.001

iCa = ionized calcium; tCa = total calcium.

CHAPTER 4
TOTAL CALCIUM CONCENTRATION IN SERUM OF HOLSTEIN DAIRY BULLS DURING THEIR FIRST MONTH OF LIFE: CHARACTERIZATION AND ASSOCIATION WITH DISEASE

Introduction

Substantial descriptions of blood total calcium levels in calves can be found in the scientific literature. All of them agree that calves at birth have higher levels of total calcium than adult cows, and that calcium levels in the calf gradually decrease until they reach the levels found in the adult animal (Agnes et al., 1993, Cabello and Michel, 1977, Dubreuil and Lapierre, 1997, Garel and Barlet, 1976, Mohri et al., 2007, Szenci et al., 1994).

Compared to the abundant knowledge of the importance that calcium homeostasis has in the cow (Curtis et al., 1983, Goff and Horst, 1997, Horst et al., 1990, Massey et al., 1993, Risco et al., 1994, Risco et al., 1984, Whiteford and Sheldon, 2005), the consequences that deviations in the level of calcium could have in the calf are not known. For instance, clinical hypocalcemia is a worldwide disease and subclinical hypocalcemia has been reported as a factor associated with several postpartum pathologies in the cow.

In the dairy industry, probably one of the most complicated challenges that producers face is the raising of young calves. As part of the future population of the farm, or just as a business in which the goal is to sell animals of quality, the performance of the future producing animal may be greatly impacted by the occurrence of disease in their early days of life (van der Fels-Klerx et al., 2001, Waltner-Toews et al., 1986). Health of animals is also an important animal welfare issue.

Many factors are related to calf disease incidence, the most important of which is the acquisition of passive immunity through colostrum (Donovan et al., 1998, Svensson et al., 2003). Besides the importance of having an adequate colostrum program in place on the farm, other

measures such as cleanliness of calf housing, provision of adequate nutrition to the calf and adequate immunization are the cornerstones of any program developed for a successful calf raising facility (Svensson et al., 2006).

Even as ever higher standards of animal care are being applied in animal production, there continues to be a high incidence of disease and mortality in the preweaning and postweaning period of dairy calves, even in farms where colostrum, nutrition and immunization programs are maximized (Gulliksen et al., 2009).

The outcome of any infectious disease is dependent on the pathogen, environment and individual factors. Therefore, there must be individual factors that predispose some calves to develop clinical infection when their healthy herdmates are exposed to the same pathogens and environment.

The hypothesis of this study is that low serum total calcium will increase the risk of disease in Holstein dairy bulls during the first forty days of life.

The goal of this study was to gather data regarding the relevance of levels of total serum calcium to incidence of calfhood disease. Calcium is a focus in this study because of its importance as a second messenger in the immune response.

In fulfillment of this goal, the first aim was to characterize serum total calcium and serum albumin during the first month of life in Holstein dairy bulls. The second goal is to determine if there is an association between serum total calcium during the first 28 days of life and the risk of disease in the first forty days of life. The third goal is to determine if there is an association between serum total calcium concentration in calves at birth with calcium concentration in their dams, and at day 2 of age with the concentration of calcium present in the colostrum they receive.

Materials and Methods

Animals

Thirty-four male Holstein calves were selected for this study.

Selection: inclusion and exclusion criteria

For enrollment of animals in the study the following criteria was set for inclusion and exclusion of animals.

Inclusion Criteria: bull calves from a normal parturition (calving difficulty of 1 or 2 of a 1 to 5 scale; that is, little or no assistance was provided at birth).

Exclusion Criteria: weak bull calves at time of first bleeding, death during the first 24h following parturition (stillbirths), calf size 1 and 5 (on a scale from 1 to 5; 1 being calves that are markedly smaller than normal and 5 being calves that are much larger than normal size); premature calves (calves born > 10 days before expected birthdate), calves resulting from induced parturition or calves with treatments for diarrhea or dehydration.

To determine if a calf was prematurely born, the date of birth was compared with the expected date of parturition of the dam. All breeding and pregnancy diagnosis data were extracted from on-farm computerized dairy management software PCDART Software (Dairy Record Management Systems, Raleigh, NC). Calving difficulty scores and calf size were recorded by trained farm personnel shortly after birth.

Animal management

Calves were managed according to the farm's written standard operating procedures (SOP) manual. Briefly, liveborn calves were fed 1.9 L (2 quarts) of high quality, refrigerator-stored colostrum and the umbilical stump was disinfected with betadine solution within 1 hr of birth. Calves were then placed individually into a clean 1m x 1.5m covered hutch for the first 21 days of life. Calves receive another 1.9 L of good quality colostrum at their second feeding (within 8

hrs of birth). The colostrum that the calf received was not from its dam. The majority of colostrums fed were from a cow that calved within 96 hrs of the calf's birth, and were stored at 4 ºC until being fed. When the demand of colostrum was higher than the storage of it, frozen colostrum was thaw at 37 ºC to feed the calves. Calves were fed 3 L of high quality (20% crude protein, 20% fat) milk replacer twice daily through 21 days of age. From day 21 through 8 weeks, calves were housed in groups of twelve animals and fed 4 L of this same milk replacer twice a day. From day 3 of life, calves were offered fresh good quality starter grain ad libitum.

Sampling Protocol

Calves were bled seven times during the study period. The first sample was taken when the calf was born, just before first colostrum feeding. Subsequent samples were taken on day two of age (between thirty-six and sixty hours of life), day five, day eleven, day fifteen, day twenty-one (±1 day) and day twenty-eight (± 2 days) of age.

Calves were bled via jugular venipuncture using a 10 cc blood collection tube without additive (BD Vacutainer®). Samples were stored at 4 ºC and within 2 hours of collection the samples were centrifuged at 1800 rpm for 15 minutes, serum collected and stored at -20 ºC until further processing.

Serum measurements: Total calcium and albumin concentrations were analyzed by the Clinical Pathology Service at the College of Veterinary Medicine of University of Florida using the procedure reported in the previous chapter.

Serum total protein concentration (measured in g/dL) using a refractometer, and IgG (mg/dL) using a single radial immunodiffusion kit (SRID, VMRD Inc.) were measured on day 2 samples only.

Health Monitoring Protocol

In the afternoons, when blood samples were collected, the health status of all calves was evaluated by a veterinarian and a trained assistant. Additionally, all calves were monitored daily just after morning feeding for clinical signs of disease by trained herd personnel with several years of experience working with dairy calves. When needed, calves were started on a specific treatment for the condition, following the SOP of the farm. The diagnosis made and the treatment given was recorded on a daily treatment sheet.

We performed a physical examination on all calves that appeared sick and on the calves that were started on treatment in the morning. The initial physical exam consisted of: temperature, heart rate, respiratory rate, auscultation of lung sounds, palpation of the umbilicus and leg joints; attitude, appetite, fecal consistency (0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces) (Walker et al. 1998) and hydration. Additionally, calves that were on treatment were daily monitored for temperature, appetite, hydration status and fecal consistency until the treatment protocol was finished. Finally, fecal consistency was scored in all calves, independently of health status, during the period of time that they spent in the individual housing system (from birth to approximately 21 days).

Sick calves were defined as those between 2 and 40 days of age with one or more of the following characteristics: fever (T >103.0 ºF), depression, partial to complete anorexia, diarrhea (length and severity), dehydration, cough, abnormal lungs sounds, umbilical infection, septic arthritis or otitis (Walker et al., 1998). Any calf with confirmed clinical signs of disease was treated according to the farm SOP and was allocated to the group of 'sick animals'.

Sample Size Calculation and Case Selection

The sample size was calculated using the means and standard deviations of previous studies (Agnes et al., 1993, Cabello and Michel, 1977, Dubreuil and Lapierre, 1997, Garel and Barlet, 1976, Mohri et al., 2007, Szenci et al., 1994). The goal was to determine a difference of 0.15 mmol/L (0.6 mg/dL) in serum total calcium between calves with clinical signs of disease and calves that did not show any sign of disease, difference that was previously reported by Cabello and Michel (1977). The standard deviation we suspected from the data of previous studies was 0.08 mmol/L (Agnes et al., 1993, Cabello and Michel, 1977, Dubreuil and Lapierre, 1997, Garel and Barlet, 1976, Mohri et al., 2007, Szenci et al., 1994). Using these values in Win Episcope 2.0, for one-tailed analysis, the sample required was twenty-four calves per group. Due to the repeated measures design of the study, the sample size could be reduced by thirty-three percent, obtaining a needed sample of sixteen calves per group (Overall and Doyle, 1994, Vickers, 2003).

The historical prevalence of calfhood disease on the study farm was twenty percent, so in order to obtain sixteen diseased calves we calculated that we were going to need to enroll eighty calves in the study.

From all enrolled animals, calves that had full sample information were the ones that were included in the study. A total of seventeen healthy calves and another seventeen calves with clinical signs of disease were suitable for analysis. Healthy calves (controls) were those calves that did not have any treatment recorded during the study period. Sick calves were calves that presented with a clinical diagnosis of otitis media, navel infection or respiratory disease. Calves that had recorded treatments for diarrhea or dehydration were not selected to form part of the healthy or the sick groups.

Other Samples and Data Collected

A blood sample was collected from dams of all enrolled bull calves within 24 hrs of parturition via coccigeal venipuncture using a 10 cc blood collection tube without additive (BD Vacutainer®). The sample was processed and total calcium and albumin was determined using the same methods as for calf samples.

A sample of the colostrum fed to calves, for both first and second feeding, was obtained prior to being fed. These samples were stored at -20 ºC until processing at the Clinical Pathology Service at UF College of Veterinary Medicine.

Additional information obtained was: data regarding birth events (calving difficulty, parity of dam, single or twin), colostrum management (time from calving to colostrum feeding, quality of colostrum received, parity of cow supplying colostrum) and calf size at birth.

Statistical Analysis

All analyses were done using SAS 9.2 (SAS Institute Inc.) software and statistical significance was stated at a p-value of < 0.05.

Descriptive analysis was performed for variables in all calves, in sick calves, and in healthy calves. Mean values with their standard error were calculated. All continuous variables were tested with the Shapiro-Wilk test for normality. To test the difference between healthy and sick calves, the Mann-Whitney test for independent nonparametric samples was used, due to the lack of normality of some variables.

Due to the anticipated influence of passive transfer of immunity through colostrum on the incidence of disease, we tested the independency of passive immunity with disease outcome. This was done using the Fisher's exact test to test independency in small sample sets.

Correlation between serum total calcium at the different sample times and the correlation of serum albumin with serum total calcium on the same sampling day were studied using Spearman's correlation test.

To accomplish the first objective of the study to analyze the variation of serum total calcium and albumin during the first 28 days of life in calves, a repeated measures analysis was performed using PROC MIXED. The dependent variable was serum total calcium or albumin, and the independent variables explored were time, group (sick or healthy) and albumin (or total calcium) and its interactions. The labels used for total calcium throughout the study are:

- Serum total calcium at birth before colostrum intake (tCa0).
- Serum total calcium between 36 and 66 hours of age (tCa2).
- Serum total calcium at five days of age (tCa5).
- Serum total calcium at eleven days of age (tCa11).
- Serum total calcium at fifteen days of age (tCa15).
- Serum total calcium at twenty-one (±1) days of age (tCa21).
- Serum total calcium at twenty eight (±2) days of age (tCa28).

For grouping, class variables used were:

- Group (0 if sick, 1 if healthy).
- Failure of transfer of passive immunity (FPT) (0 if IgG ≥ 1000mg/dL, 1 if IgG < 1000mg/dL) (Ameri and Wilkerson, 2008).

To determine the effect of calcium on disease incidence, we used only those calves treated for respiratory disease and otitis, as these two conditions accounted for 88% of disease diagnoses. Sick calves were categorized as normocalcemic if the serum sample taken on the sampling day immediately before diagnosis was equal to or above the mean of that sampling day. If that sample was below sampling day mean, the calf was categorized as hypocalcemic. For

almost all ‘sick calves’ sample day used was day 15, day 21 or day 28. For healthy calves, each calf was categorized as hypo- or normocalcemic at sample day 15, 21 and 28 following the same criteria described above. If the calf was hypocalcemic on two of those three days it was categorized hypocalcemic; if it was hypocalcemic on one of those three sampling days it was categorized as normocalcemic. Odds ratios were calculated using WinEpiscope 2.0 Software.

The relationship between total calcium in the dam, dam parity and total calcium in the first sample taken from the calf (tCa0) was also analyzed. The relationship between serum total calcium on day 2 (tCa2), total calcium present in the colostrum (first and second feedings) and parity of the dams that where the donors of the colostrum were also evaluated. To accomplish this objective, Spearman correlation was performed using PROC CORR. Following the correlation, those variables that presented an association were studied in simple regression and finally a model was built using backward elimination, with p-value to enter to the model set at 0.20 and to stay at 0.05.

Results

Descriptive Analysis

Serum total calcium peaked in the calf at birth (3.24 mmol/L), decreased until day 11 and remained stable from 11 to day 28 (Table 4-1; Figure 4-1). Mean albumin values were between 2.60 to 2.79 g/dL throughout the study period. Total calcium in colostrum had little variation ranging from 10.85 to 11.45 mmol/L (Table 4-1). The test for differences in serologic mean values between healthy calves and calves diagnosed sick, found serum total calcium at 28 days significantly ($p = 0.011$) higher in control calves compared to calves that were sick (Table 4-2). The mean fecal score for all calves was 2, and was not different for healthy calves and sick calves during the first 21 days of life ($p = 0.837$). Serum IgG and the proportion of calves with failure of transfer of passive immunity (IgG < 1000 mg/dL) was not different between controls

and sick calves (p = 0.805). The proportion of calves with failure of passive transfer was 33% in all calves; 31% in sick calves and 35% in controls.

From the seventeen calves in the sick group, the mean age at onset of clinical disease was 25.4 days of age, the youngest calf being 11 days of age and the oldest 37 days. Navel infection was diagnosed in the youngest calves, with a mean age at diagnoses of 13 days. Respiratory infection and otitis media were diagnosed in older calves, at 26 and 27.5 days of age respectively (Table 4-3).

Repeated Measures Calcium and Albumin

During the first days of life there was a significant correlation in serum total calcium with the previous sample. This correlation decreased in magnitude as age increased, and by day 11 there was no significant correlation with the previous sample. Serum albumin correlated in all sample times with serum total calcium, but the correlation was of greater magnitude from day 15 until the end of the sampling period at day 28 (Table 4-4).

In the repeated measures analysis, serum total calcium could be explained by time of sampling (p = <0.0001) and albumin (p = <0.0001). Classification group (healthy or sick) did not explain the variation in calcium (p = 0.140), but the interaction of group and time had a p = < 0 .0001. This could be interpreted as that the concentration in serum tCa was similar between healthy and sick calves, but the change in calcium concentration in each time period is different in healthy calves compared to sick ones. If the effects of albumin and time were together in the model, both remained with the same significance, but their interaction did not help explain the variation of serum total calcium. Figure 4-1 and Figure 4-2 graphically presents total calcium levels in calves over time in all animals, and in healthy vs. sick calves.

Time was also significant in the model of serum albumin in calves (p = 0.013) as was calcium (p = 0.001). Group classification as sick or healthy was not significant in explaining

albumin variation (p = 0.561), but the interaction between group and time was marginally significant (p = 0.068). Calcium, time and the interaction of calcium and time were significant in the model describing the albumin in these study calves. The variation of serum albumin with age in all animals, and in healthy vs. sick calves, is presented in Figure 4-3 and Figure 4-4.

Calcium and Disease Association

The clinical diseases that were diagnosed in the 17 sick calves were navel infection, respiratory disease and otitis media (Table 4-3). As calves with otitis and respiratory disease had a similar age distribution and historically have had a common etiology in the study herd (*Mycoplasma bovis*), they were grouped for this calculation. They also accounted for >88% of disease diagnoses. Only two calves were diagnosed with navel infection, which precluded further analysis of its association with serum total calcium (Table 4-5).

In the analysis of the association between serum total calcium and otitis/respiratory infection, calves that were classified as below the mean of serum total calcium were 2.10 times more likely to subsequently be treated for these conditions than calves that were above the mean (Table 4-6). This association was not statistically significant (95% CI = 0.49 – 9.00), but the confidence interval disclosed that calves with serum calcium below the mean for their age could be up to 9 times more likely to be diagnosed with otitis/respiratory infection compared to calves with normal calcium.

Calcium at Birth and its Relation to the Dam

The Spearman's correlation matrix (Table 4-7) shows positive significant correlations between serum total calcium of the calf at birth with dam total calcium (p=0.008). There was also a significant negative association (p=0.005) between serum total calcium of the dam and her parity. Between colostrum characteristics and serum total calcium on day 2, significant positive correlation was only found between total calcium in second colostrum fed and serum total

calcium on day 2 (p=0.028) (Table 4-8). In the regression analysis, the variable that fitted the best model to predict calf total calcium at birth was serum total calcium in the dam, with a p-value of 0.006, and serum total calcium on day 2 was predicted only by total calcium in second colostrum fed (p = 0.020) (Table 4-9).

Discussion

Total calcium concentration in calf serum has its greatest value at birth, as it has been previously reported (Agnes et al., 1993, Cabello and Michel, 1977, Mohri et al., 2007, Szenci et al., 1994). In my study, total calcium is higher than those reported by Szenci et al. (1994) and Mohri et al. (2007), but sampling dates are not fully comparable. Serum albumin were very stable during the twenty-eight days of the sampling period compared to the study presented by Naussbaum et al. (2002) that reported an elevation in plasma albumin between seven and fourteen days of age. Mohri (2007) also reported an increase in albumin in calves from birth to day forty-two of age.

When serum values of healthy and sick calves were compared significant differences were only found in serum total calcium on day 28. Cabello and Michel (1977) found an almost constant difference in serum tCa between their healthy calves and calves with diarrhea during the twenty days that their study lasted. In their study there was a significant difference in globulins (measured as the difference between total protein and albumin) between their two groups on day one and 2 of life, being a possible confounder of their results. In the present study, neither the concentration of IgG nor the proportion of calves with failure of the transfer of passive immunity was different between groups, although the overall proportion of animals that failed to obtain good passive transfer of immunity was higher than expected.

The association between disease and low calcium found in calves with respiratory infection and otitis has not been reported before but, to my knowledge, the only study that tried to explain

a relationship between serum total calcium and occurrence of disease is that reported by Cabello and Michel (1977). In the present study, I made every attempt to appropriately define the time relationship between low calcium and disease presentation by using the calcium measures from the sampling day immediately prior to disease diagnosis. For healthy calves this was more problematic because several sampling days from each calf were used in the analysis. One possible reason for the lack of statistical significance in the association between calcium and clinical signs of disease is that the sample size needed to find an association was greater than what I had. The odds ratio of 2.10 suggests that calcium may be an important risk factor for otitis media and respiratory infection in pre-weaned dairy calves, and deserves further investigation.

The correlation between serum total calcium and albumin in the same sample could explain the effect that serum albumin has over serum tCa. An increase in serum albumin could increase the number of calcium molecules present in blood that would bind to serum albumin, producing a temporary electrostatic imbalance between the intravascular and extravascular spaces. This imbalance would be restored by ion exchange between both spaces due to the Donnan effect, producing an increased influx of calcium from the extravascular space into the blood stream (Fogh-Andersen et al., 1993). As result of this calcium influx, serum total calcium will be increased and ionized calcium would be modified little. Albumin physiologically increases in the calf during the first forty days of age (Knowles et al. 2000, Mohri et al. 2007), but our data did not show this increase in albumin with age.

The association between dam parity and serum total calcium in the cow has been widely documented and results from lower capacity for calcium mobilization and absorption as the age of the cow increases (Horst et al., 1990). The results of this study confirm a negative relationship between parity and cow total calcium. Studies of the relationship between cow and calf calcium

are lacking in the scientific literature, but Szenci et al. (1994) did not find a positive association between calcium in cows and their offspring, while Kume and Tanabe (1993) found an association between cow parity and calcium in the calf. In my study I have found a significant relationship between serum total calcium in the calf at birth and dam serum total calcium. Also an association was found between serum total calcium on day 2 and total calcium concentration of the second colostrum fed to the newborn. Colostrum total calcium did not show any correlation with cow parity in this study, although it has been reported that calcium in colostrum is higher in first and second lactation and decreases as lactation number increases (Kume and Tanabe, 1993). The relation between total calcium in colostrum and serum total calcium in the calves after being fed was not reported in that study. It is unlikely that calcium intake with colostrum would have any physiologic effect two days after ingestion but it could be related to other factors not studied in the present study.

Conclusion

Serum total calcium at birth can be explained by dam serum total calcium, and colostrum calcium concentration may have an influence on calf serum total calcium in the early days of life. After the initial high levels of total calcium there is a decrease in its concentration. Disease risk based on serum total calcium was not fully determined, but the moderately strong, non-significant association between respiratory disease or otitis and serum total calcium warrants further investigation.

Table 4-1. Descriptive statistics of selected blood values, colostrum total calcium concentration and fecal scores in a study of calcium in neonatal calves.

Group	All				
	N	Mean	Min	Max	SEM
IgG (mg/dL)	33	1584	557	3170	138.99
TP (g/dL)	33	6.1	4.3	7.3	0.13
Total calcium 1st colostrum (mmol/L)	30	11.24	10.85	11.45	0.02
Total calcium 2nd colostrum (mmol/L)	25	11.20	10.85	11.43	0.03
Dam total calcium (mmol/L)	33	2.02	1.60	2.53	0.05
Dam albumin (g/dL)	33	3.15	2.60	3.60	0.04
Age diagnosed sick (days)	17	25	11	37	2.18
Total calcium day 0 (mmol/L)	33	3.24	2.28	4.15	0.06
Total calcium day 2 (mmol/L)	33	3.16	2.53	3.70	0.05
Total calcium day 5 (mmol/L)	34	3.03	2.65	3.45	0.03
Total calcium day 11 (mmol/L)	34	2.55	1.65	3.63	0.06
Total calcium day 15 (mmol/L)	34	2.57	1.98	3.08	0.04
Total calcium day 21 (mmol/L)	34	2.57	2.33	2.98	0.03
Total calcium day 28 (mmol/L)	34	2.54	2.10	2.85	0.03
Albumin day 0 (g/dL)	33	2.76	1.90	3.50	0.05
Albumin day 2 (g/dL)	33	2.64	2.20	3.10	0.04
Albumin day 5 (g/dL)	34	2.64	2.40	2.90	0.02
Albumin day 11 (g/dL)	34	2.69	1.60	4.20	0.08
Albumin day 15 (g/dL)	34	2.60	2.00	3.40	0.05
Albumin day 21 (g/dL)	34	2.79	2.10	3.20	0.04
Albumin day 28 (g/dL)	34	2.71	2.20	3.30	0.05
Fecal score mean	34	2.00	1.50	2.80	0.05

Fecal score mean = mean fecal score from day 1 to day 21.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces) (Walker et al. 1998).

Table 4-2. Mann-Whitney test for difference in calcium and albumin concentration variable means between healthy and sick calves.

Group	Healthy		Sick		
	Mean	SE	Mean	SE	p-value
IgG (mg/dL)	1541	206.74	1629	190.50	0.683
TP (g/dL)	6.0	0.20	6.1	0.16	0.709
Total calcium 1st colostrum (mmol/L)	11.23	0.04	11.26	0.03	0.680
Total calcium 2nd colostrum (mmol/L)	11.15	0.04	11.27	0.04	0.062
Dam total calcium (mmol/L)	2.05	0.06	2.00	0.07	0.557
Dam albumin (g/dL)	3.14	0.05	3.15	0.07	0.817
Total calcium day 0 (mmol/L)	3.24	0.08	3.23	0.08	0.557
Total calcium day 2 (mmol/L)	3.18	0.07	3.15	0.06	0.790
Total calcium day 5 (mmol/L)	2.99	0.05	3.07	0.03	0.067
Total calcium day 11 (mmol/L)	2.49	0.06	2.62	0.11	0.413
Total calcium day 15 (mmol/L)	2.60	0.04	2.54	0.07	0.540
Total calcium day 21 (mmol/L)	2.62	0.04	2.52	0.03	0.160
Total calcium day 28 (mmol/L)	2.63	0.04	2.45	0.05	0.011
Albumin day 0 (g/dL)	2.75	0.05	2.78	0.09	0.901
Albumin day 2 (g/dL)	2.62	0.06	2.67	0.06	0.736
Albumin day 5 (g/dL)	2.62	0.04	2.66	0.03	0.540
Albumin day 11 (g/dL)	2.59	0.09	2.78	0.13	0.496
Albumin day 15 (g/dL)	2.61	0.06	2.59	0.08	0.734
Albumin day 21 (g/dL)	2.81	0.06	2.76	0.06	0.518
Albumin day 28 (g/dL)	2.79	0.06	2.63	0.07	0.092
Fecal score mean	2	0.07	2	0.07	0.837

Fecal score mean = mean fecal score from day 1 to day 21.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces) (Walker et al. 1998).

Table 4-3. Age distribution of disease diagnosis in sick calves.

Disease	N	Mean	Minimum	Maximum	Std.Dev.	SE
Otitis media	11	27.5	15	37	8.8	2.6
Navel infection	2	13.0	11	15	2.8	2.0
Respiratory disease	4	26.0	15	31	7.4	3.7

Table 4-4. Spearman's correlation matrix and accompanying p-value between total calcium at all sampling times and with albumin on the same sample day.

	Statistic	tCa 0	tCa 2	tCa 5	tCa 11	tCa 15	tCa 21		ALBUMIN
tCa 0	r							0.365	Albumin 0
	p							0.037	
tCa 2	r	0.605						0.598	Albumin 2
	p	0.000						<0.001	
tCa 5	r	0.467	0.354					0.350	Albumin 5
	p	0.006	0.043					0.043	
tCa 11	r	0.349	0.359	0.243				0.535	Albumin 11
	p	0.046	0.040	0.166				0.001	
tCa 15	r	-0.046	-0.262	-0.156	0.014			0.709	Albumin 15
	p	0.801	0.140	0.377	0.936			<0.001	
tCa 21	r	0.181	0.150	-0.255	0.096	0.321		0.676	Albumin 21
	p	0.314	0.403	0.146	0.589	0.065		<0.001	
tCa 28	r	-0.097	0.085	-0.145	-0.179	-0.068	-0.097	0.710	Albumin 28
	p	0.591	0.638	0.411	0.311	0.704	0.583	<0.001	

tCa_n = serum total calcium on test day.

Table 4-5. Contingency tables of healthy calves and calves with navel infection vs. serum calcium.

DAY 11	Calcium < mean	Calcium ≥ mean
Navel Infection	2	0
Healthy	8	9
DAY 15	Calcium < mean	Calcium ≥ mean
Navel Infection	2	0
Healthy	8	9

Calcium < mean = serum total calcium lower than the mean value for all animals in this study at the specific sample age.
Calcium ≥ mean = serum total calcium greater than the mean value for all animals in this study at the specific sample age.

Table 4-6. Contingency tables of healthy and sick (otitis and respiratory infection) calves vs. serum calcium in the sample collected the day immediately before diagnosis.

	Calcium < mean	Calcium ≥ mean	OR	95% CI
Sick	7	8	2.10	0.49-9.00
Healthy	5	12		

Calcium < mean = serum total calcium lower than the mean value for all animals in this study at the specific sample age.

Calcium ≥ mean = serum total calcium greater than the mean value for all animals in this study at the specific sample age.

Table 4-7. Spearman's correlation matrix and accompanying p-values between serum total calcium at birth in the calf, dam parity and dam calcium at calving.

	Statistic	Dam parity	Dam total calcium
Dam total calcium	r	-0.478	
	p	0.005	
tCa 0	r	-0.236	0.459
	p	0.185	0.008

tCa 0 = serum total calcium at birth.

Table 4-8. Spearman's correlation matrix and accompanying p-values between calcium at 2 days of age, colostrum calcium, and parity of the cow donor.

	Statistic	tCa 2	1st Colostrum Parity	2nd Colostrum Parity	1st Colostrum tCa
1st Colostrum Parity	r	-0.149			
	p	0.439			
2nd Colostrum Parity	r	0.080	-0.116		
	p	0.709	0.599		
1st Colostrum tCa	r	0.244	-0.274	0.020	
	p	0.202	0.151	0.926	
2nd Colostrum tCa	r	0.578	-0.180	-0.010	0.256
	p	0.003	0.410	0.964	0.228

tCa = total calcium.
tCa 2 = serum total calcium at 2 days.

Table 4-9. Linear regression analysis estimates to model calf serum total calcium at birth as a function of dam tCa and at day 2 as a function of colostrum total calcium.

Dependent variable	Independent variable	DF	Estimate	SE	t Value	p-value
Serum total calcium day 0	Intercept	1	2.013	0.419	4.81	<0.001
	Dam Total Calcium	1	0.608	0.207	2.94	0.006
Serum total calcium day 2	Intercept	1	-27.973	16.226	-1.72	0.099
	2nd colostrum total calcium	1	0.909	0.362	2.51	0.020

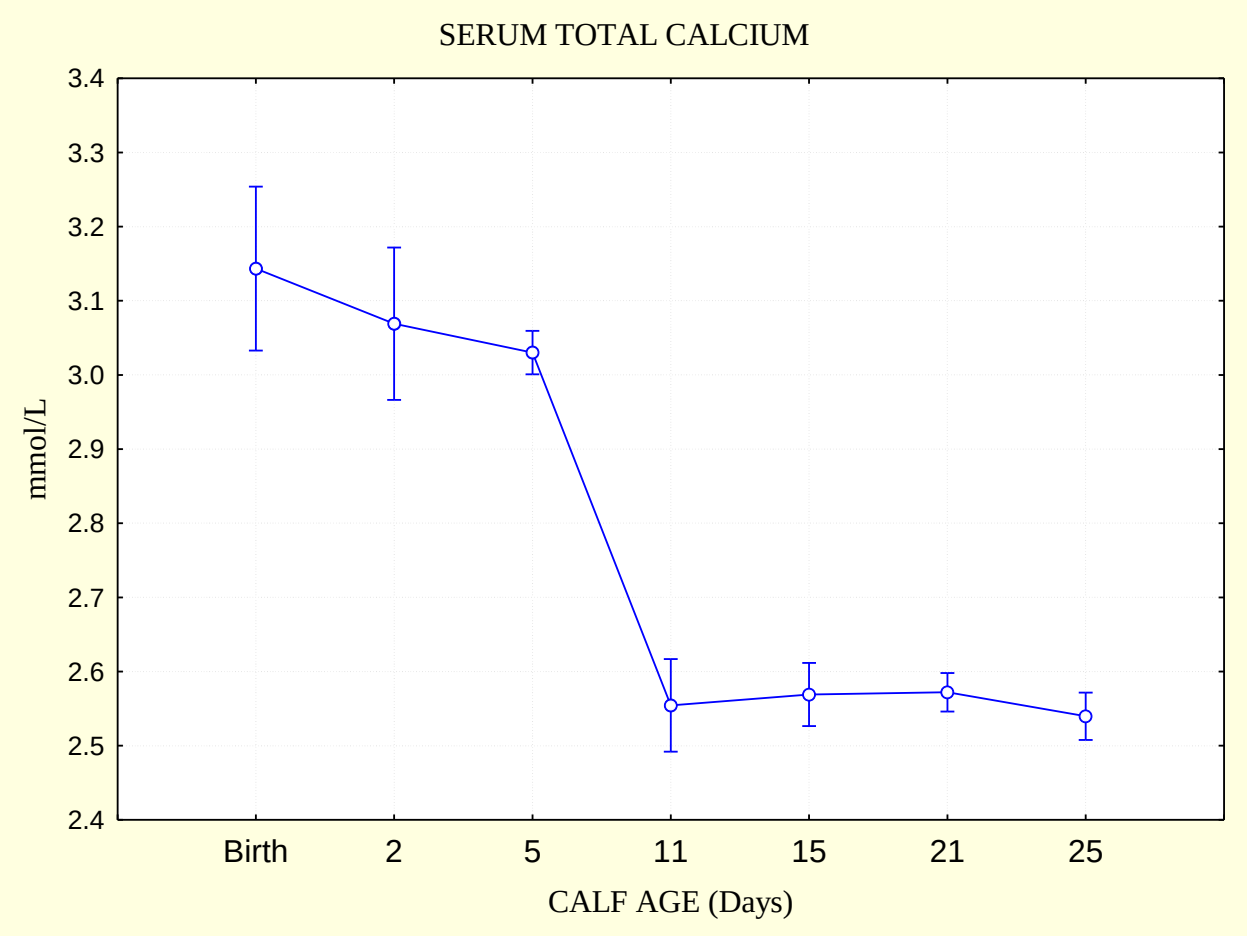

Figure 4-1. Serum total calcium concentration means ± standard errors from birth to 28 days of age in all study calves.

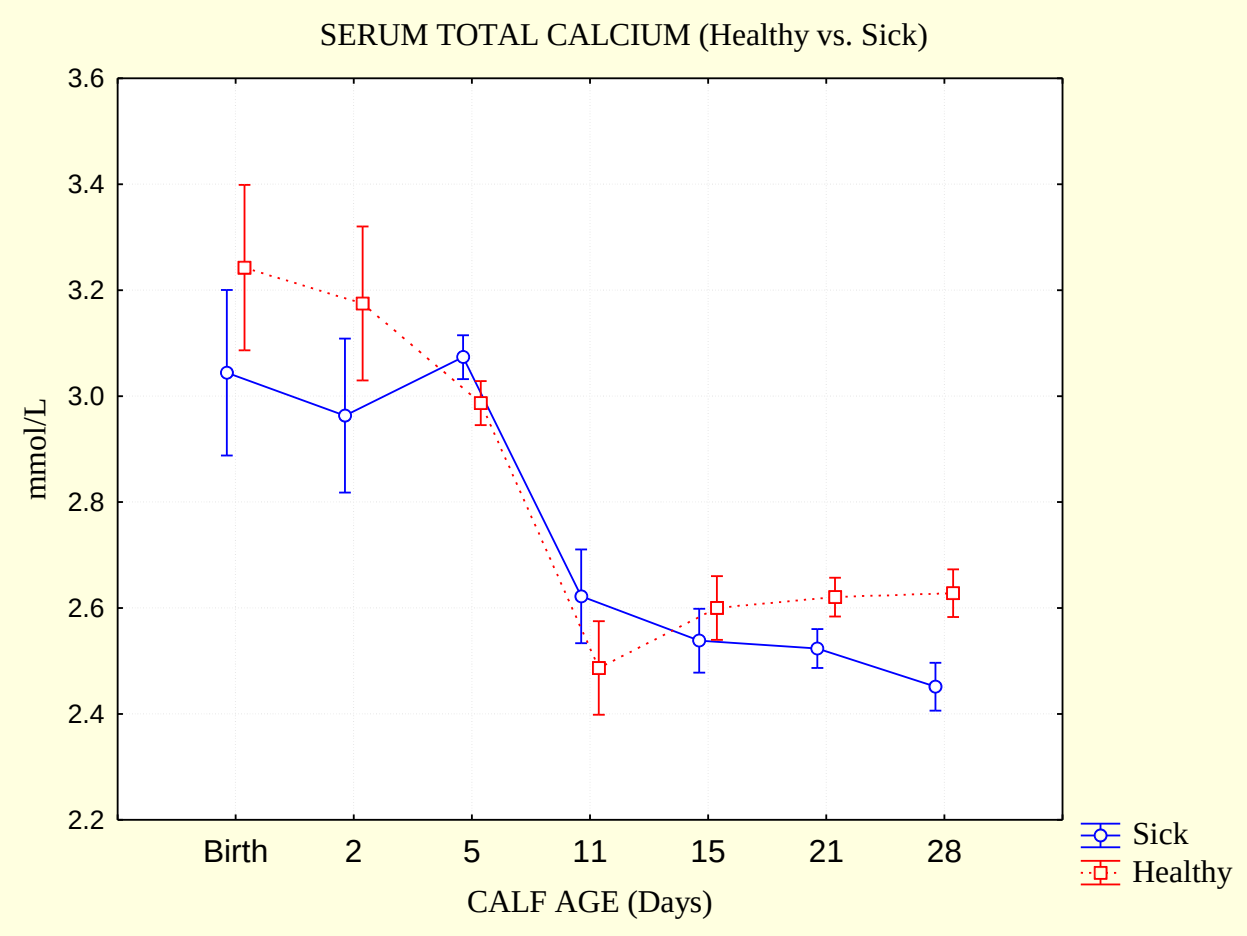

Figure 4-2. Serum total calcium means ± standard errors from birth to 28 days of age, in healthy and in sick calves.

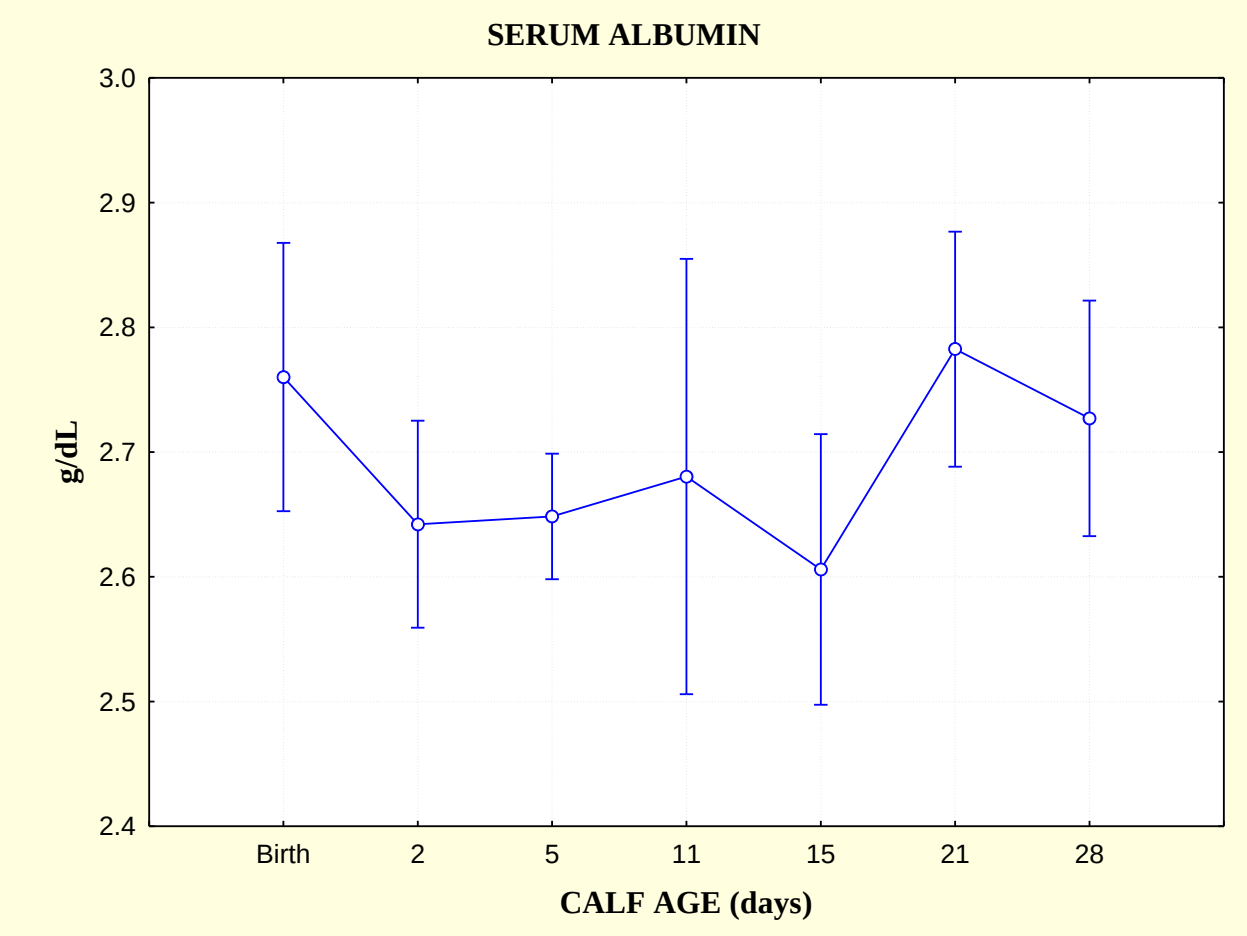

Figure 4-3. Serum albumin means ± standard errors from birth to 28 days in all study calves.

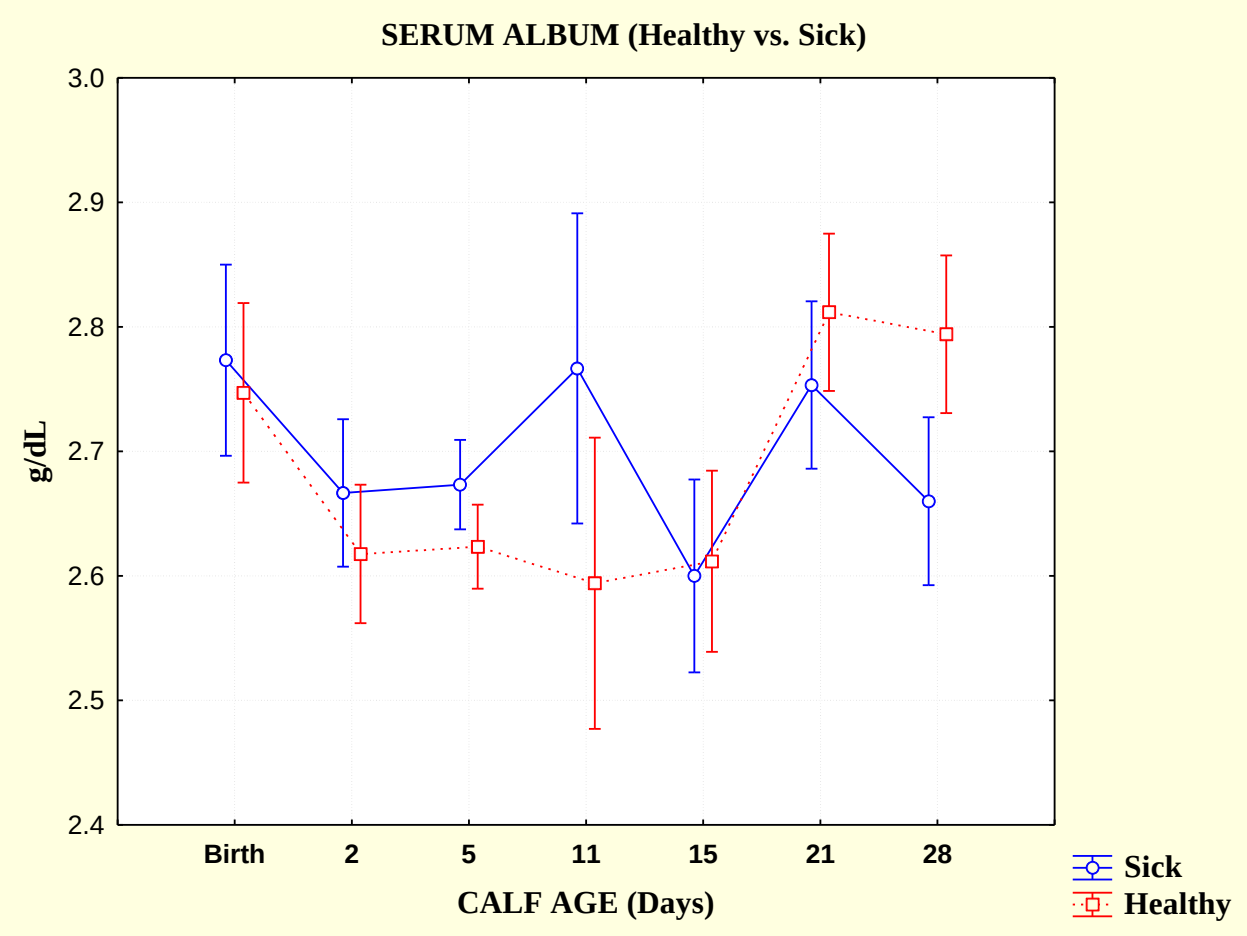

Figure 4-4. Serum albumin means ± standard errors from birth to 28 days, in healthy and in sick calves.

CHAPTER 5
FLOW CYTOMETRY AND CYTOKINES: ASSOCIATION BETWEEN SERUM BLOOD CALCIUM CONCENTRATION AND IMMUNE RESPONSE IN CALVES

Introduction

As part of the innate immune system, neutrophils are present in the calf at birth and constitute the first line of defense when a pathogen enters into the body. Monocytes are also present at this age, and both can be stimulated and can phagocytize bacteria (Kampen et al., 2006, Menge et al., 1998). The functions of these cells are to phagocytize and destroy the invading microorganism thus neutralizing the infectious process. To achieve their goal, these cells need to be activated by surface receptors which lead to a complex intracellular signaling process in which ionic calcium plays an important role. Phagocytosis and oxidative burst have been associated with intracellular calcium influx and extracellular calcium levels *in vitro* (Higuchi et al., 1997a, Higuchi et al., 1997b, Ortiz-Carranza and Czuprynski, 1992, Zheng et al., 1992). Cytokine production is another response of leukocytes to various stimuli, and calcium also plays an important part in cytokine production (Brown et al., 2004, Di Sabatino et al., 2009, Liu et al., 2008).

The association between low serum total calcium in cows with clinical hypocalcemia, and a reduction in the influx of calcium into their neutrophils, compared to normocalcemic periparturient cows have been shown in clinical studies (Kimura et al., 2006). In the calf, the relationship between serum total calcium and the capacity of neutrophils and monocytes to become stimulated, begin phagocytosis of bacteria and produce oxygen reactive species (ROS) is unknown. Also, it is not known whether or not serum calcium levels influence the ability of the calf's leukocytes to produce cytokines under a bacterial stimulus.

The hypothesis of the present study is that calves with higher serum total calcium compared to their herdmates, will have a stronger early immune response to bacterial stimuli,

measured as higher activation of neutrophils and monocytes and higher production of TNF-alpha and IFN-gamma compared to calves with lower serum total calcium.

The goal of this study was to explore the association between serum total calcium and neutrophil and monocyte activation, measured as the proportion of activated cells after stimulation with bacteria, and the ROS production by phagocytes, measured as the mean fluorescence emitted by phagocytes. The second objective is to explore the association between serum total calcium and production of interferon gamma (IFN-gamma) and tumor necrosis factor alpha (TNF-alpha). Both of these objectives will be studied in Holstein bulls at 2 and 21 days of age.

Materials and Methods

Animals

Fourteen male Holstein calves were selected to conduct this study. Calves were those from the study described in Chapter 4. The selection of these calves was done by convenient sampling. These were the calves that were enrolled at the end of the study. At enrollment they were all healthy calves, but during the study period some remained healthy, some presented more severe diarrhea and some were diagnosed with otitis media and respiratory infection. Management procedures are also those described in Chapter 4.

Sampling Protocol and Processing Methods

At 2 and 21 (±1) days of age, blood samples were taken to determine phagocytic cell function (using flow cytometry), serum total calcium and albumin. All blood samples were collected in the afternoon, before the calves received their second daily feeding of milk.

Calves were bled via jugular venipuncture using one 10 cc blood collection tube without anticoagulant and another 10 cc blood collection tube with lithium heparin (BD Vacutainer®). Blood was collected and handled with care to avoid hemolysis.

Blood processing for chemical analysis

Blood samples for calcium and albumin determination were collected in tubes without anticoagulant and stored at 4 ºC until further processing. Within 2 hours of collection, the samples were centrifuged at 1800 rpm for 15 minutes. The serum was collected and stored at -20 ºC until laboratory processing of the sample. Chemical analyses were performed by the Clinical Pathology Service, College of Veterinary Medicine of the University of Florida. The methodology to determine serum total calcium and albumin is described in Chapter 3. Immunoglobulin G was also determined at day 2 of life as described in Chapter 3.

Blood processing for flow cytometry

Blood collected in heparinized tubes was gently agitated and left at room temperature in a horizontal position during transportation from the farm to the laboratory. Blood samples were processed within 2 hours of collection.

Activation of phagocytic cells was measured using pHrodo™*E.coli* BioParticles ® Conjugate for phagocytosis (Molecular Probes ™, Invitrogen ™). A sample of the heparinized blood (100 µL) was incubated with pHrodo™*E.coli* BioParticles ® Conjugate (20 µL of reconstituted product) at 38 ºC in continuous agitation for 2 h. A control sample for each animal was used following the same process as described above but without using pHrodo™*E.coli* BioParticles ® Conjugate. After incubation, phagocytosis initiated by the presence of *E.coli* was stopped by placing the samples on crushed ice.

To eliminate the background that red blood cells (RBCs) produce in the flow cytometry, RBCs were lysed using a commercial lysing solution (BD Lysing Buffer ™). The process consisted of adding 2 ml of the lysing solution to the samples, vortexing and waiting for 5 minutes to produce the lysis of RBCs. Samples were then washed twice by adding 2 ml of DPBS to the tubes and centrifuging for 5 minutes at 2000 rpm to eliminate the lysing buffer.

Supernatant was removed by inverting the tubes. Tubes were then placed briefly on crushed ice to be taken to the laboratory to perform the flow cytometry.

Blood processing for cytokine determination

Tubes with blood containing heparin were handled as previously described for the flow cytometry. The tubes were centrifuged for 15 minutes at 2500 rpm. Buffy coat was collected and the remaining plasma was saved for later use. The buffy coat was centrifuged again for 5 minutes at 1800 rpm and the resulting buffy coat was collected and mixed with 2 ml of the autologous calf plasma that had been saved in the first centrifugation. To measure the concentration of leukocytes in the 2 mL of plasma mixed with the buffy coat, we took 20 µL of the solution and mixed it with 380 µL Turk solution, to lyse the RBCs. Leukocytes were counted in a Neubauer counting chamber.

Leukocytes were diluted in autologous plasma to a final concentration of $2x10^6$ cells/mL. Control and treated samples were incubated in a six well cell culture plate (BD Falcon™). Controls consisted of 2 mL of the final concentration of leukocytes and treated samples were 2 mL of the leukocytes stimulated with 20 µL of concavalin A (ConA). Plates were incubated for 48 h at 38 ºC and 5% CO_2 concentration.

After the incubation period, the supernatant was collected from the wells. They were centrifuged for 15 minutes at 2000 rpm, and the supernatant was collected and frozen at -20 ºC until further analysis.

Flow Cytometry

Neutrophils and monocytes were discriminated and quantified by combined measures of forward scatter (FS) which is related to the size of the cells, and side scatter (SS) that is related to the granularity of the cells (Figure 5-1). Neutrophils and monocytes were gated to FS against fluorescence cytograms (Figure 5-2), and analyzed for target fluorescence. The fluorescence

emitted by the pHrod™ dye-labeled *E. coli* bacteria has its maxima at pH = 4 and decreases as pH increases. In the flow cytometer, the fluorescence emitted by the phagocytosing cells, when they had ingested the bacteria, was collected with the FL2 channel (fluorescence emitted at 600 nm).

Control blood samples were used as baseline. The proportion of phagocytosing cells was defined as the percentage of gated cells with target fluorescence which were located in region 2 of the cytogram (see Figure 5-2). In control samples this region was set with a percentage of 0.30%±0.03 to obtain the same baseline values between animal samples. To calculate the response of neutrophils and monocytes to the bacteria added in the sample, the initial percentage of phagocytosing cells was subtracted from the percentage of phagocytosing cells in the samples exposed to bacteria. The same procedure was performed to calculate the mean of fluorescence emitted by phagocytic neutrophils and monocytes.

The variables obtained in the flow cytometry for both neutrophils and monocytes is briefly described in this list:

- Side scatter of neutrophils in controls and in samples stimulated with bacteria (SSNC and SSNS).
- Side scatter of monocytes in controls and in samples stimulated with bacteria (SSMC and SSMS).
- Forward scatter of neutrophils in controls and in samples stimulated with bacteria (FSNC and FSNS).
- Forward scatter of monocytes in controls and in samples stimulated with bacteria (FSMC and FSMS).
- Proportion of neutrophils in controls and in samples stimulated with bacteria (%NC and %NS).
- Proportion of monocytes in controls sample and in samples stimulated with bacteria (%MC and %MS).

- Proportion of phagocytizing neutrophils in controls and in samples stimulated with bacteria (%PNC and %PNS).
- Difference in the proportion of phagocytizing neutrophils between controls and samples stimulated with bacteria (D%PN).
- Proportion of phagocytizing monocytes in control samples and in samples stimulated with bacteria (%PMC and %PMS).
- Difference in the proportion of phagocytizing monocytes between controls and samples stimulated with bacteria (D%PM).
- Mean fluorescence emitted by phagocytizing neutrophils in control samples and in samples stimulated with bacteria (MFNC and MFNS).
- Difference in the fluorescence emitted by phagocytizing neutrophils between controls and samples stimulated with bacteria (DFN).
- Mean fluorescence emitted by phagocytizing monocytes in controls and in samples stimulated with bacteria (MFMC and MFMS).
- Difference in the fluorescence emitted by phagocytizing monocytes between controls and samples stimulated with bacteria (DFM).

Cytokine Determination using an ELISA

Interferon gamma (IFN-gamma)

We determined the production of IFN-gamma by leukocytes using an ELISA. Ninety-six well plates were coated using a mouse anti-bovine interferon gamma monoclonal antibody (MCA2112, ©AbD Serotec, © MorphoSys AG). Coating antibody was used in a concentration of 1 μg/mL (diluted in carbonate-bicarbonate buffer, pH = 9.6). Plates were covered and incubated overnight at 4 ºC and blocked with 1% BSA blocking buffer for 1 h at 37 ºC. Serial dilutions of recombinant IFN-gamma were used in triplicates to create the standard curve. Standards were diluted in 1:2 bovine plasma with PBS 0.05% v/v Tween 20. Recombinant IFN-gamma (Endogen®, Pierce) and calf plasma diluted in PBS-Tween (1:2 dilution) were incubated in the plate overnight at 4 ºC. Secondary monoclonal antibody (MCA1783, ©AbD Serotec, © MorphoSys AG) was added at a concentration of 1 μg/mL and incubated for 1 h at 37 ºC.

Finally, Avidin-horseradish peroxidase conjugate (BD Pharmingen ™, BD Biosciences) was added (1:1000 dilution) and incubated at room temperature in the dark for 30 minutes. Plates were washed and TMB (tetramethylbenzidine) substrate (BD OptEIA ™, BD Biosciences), was added and incubated for 20 minutes at room temperature in the dark. After 20 minutes of incubation, I added Stop Solution (BD OptEIA ™, BD Biosciences) to the wells and the plate was read using an ELISA plate reader at 450 nm.

A standard curve was obtained plotting the OD values for the known INF-gamma concentrations. An equation was created and the sample IFN-gamma concentrations (pg/mL) were obtained resolving the equation.

Tumor necrosis factor alpha (TNF-alpha)

Tumor necrosis factor alpha was determined using a similar ELISA procedure to that described above for IFN-gamma. A rabbit anti-bovine tumor necrosis factor alpha polyclonal coating antibody (Endogen®, Pierce) was used at 5 μg/mL and was incubated overnight at room temperature in a dark place. Fish skin gelatin 2% was used as blocking buffer and plates were blocked for 1 h. Standards with TNF recombinant (Endogen®, Pierce) and samples were prepared as described above for the IFN ELISA, and incubated for 1 h at room temperature. A biotinylated rabbit anti-bovine polyclonal secondary antibody (Endogen®, Pierce) at 200 μg/mL was finally added, and the subsequent steps were the same as in the IFN-gamma ELISA.

In this case due to a high background, a standard curve that was valid at low concentrations of TNF-alpha was not obtained. Instead, a sample to positive ratio (S/P) was calculated. For this task, the concentration of recombinant TNF that had the least variation between wells was the one considered as the positive sample. The variation was calculated with the coefficient of variation (CV) between the triplicates. On day 2 samples, the sample with least variation used as

the positive sample had a concentration of TNF-alpha of 1000 pg/mL (CV = 1.3) and on day 21 the positive had 62.5 pg/mL of TNF-alpha (CV = 2.2).

Statistical Analyses

All statistical analysis was performed using SAS ver 9.2 (SAS Institute).

Flow cytometry

A descriptive analysis was performed independently for day 2 and day 21 of age, and mean values and standard error of means were determined.

On day 2, the studied variables were the flow cytometry values described above, serum total calcium in the calf at birth and at day 2, serum IgG, day of diagnosis of sick calves, and fecal score on day 2. On day 21, the same variables were studied except that fecal score was considered the mean value of the first seventeen days of age, and serum total calcium on day 21 was used instead of total calcium on day 2.

Variables were treated as nonparametric due to the small sample size (n = 13 on day 2 and n = 14 on day 21). Differences between the variables obtained with the flow cytometer, comparing controls and samples with bacteria, were tested with Wilcoxon test for paired samples using PROC UNIVARIATE.

Correlation was performed using Spearman's test for nonparametric data with PROC CORR. Variables that showed significant correlation ($p < 0.05$) or trend of correlation with D%PN, D%PM, DFN or DFM were investigated in simple linear regression. The significant variables were modeled in a backward elimination procedure to create a multiple regression model, where serum total calcium at day 2 or 21, respectively, was forced in the model. To enter in the model, p-value was set at < 0.20 and to stay at < 0.05. In the cases where post-hoc power analysis was performed, it was calculated at alpha = 0.05. PROC REG was used to perform this analysis.

In order to investigate an association between tCa2 or tCa21 and responses of neutrophils and monocytes, new variables were created. Two classification categories were created from tCa2 and tCa21:

- NORMAL/HIGH calcium, if tCa was equal or greater to tCa mean.
- LOW calcium, if tCa was lower than tCa mean.

The same type of classification was done for D%PN, D%PM, DFN and DFM, for samples on day 2 and day 21:

- 1 or POSITIVE response, if the studied flow cytometry variable was equal or greater to its mean.
- 0 or NEGATIVE response, if it was lower than its mean.

The odds ratio of having a good response with normal/high calcium was compared to the odds of having a good response with low calcium. Due to the small sample size several cells had counts less than five, therefore Fisher's exact test was used for this analysis.

Based on tCa2 (tCa21) classification, Mann-Whitney test for independent samples was performed to study differences between the study variables in calves with high/normal calcium and calves with low calcium on the day of the study.

TNF-alpha and IFN-gamma

The analyses performed for the cytokines produced were as described above for analyses of flow cytometry data. The new dependent variables created in this case were:

- IFN-gamma production, calculated by subtracting IFN control from the IFN of samples with ConA.
- S/P increment, calculated by subtracting TNF-alpha S/P in controls from TNF-alpha S/P in ConA stimulated samples.

Results

Flow Cytometry

A total of 13 samples from day 2 and 14 from day 21 were analyzed. The difference in numbers is due to difficulties found in the technique on the first day of sampling resulting in low confidence in the precision of the data. Therefore data from the first calf analyzed on day 2 was discarded.

Day 2 of life

Quantification of neutrophils and monocytes: There was a significant increase in SS and FS, in neutrophils and monocytes, after bacterial stimulation (Table 5-1). The proportion of neutrophils and monocytes gated from the samples were also significantly different before and after stimulation (Table 5-1).

The proportion of neutrophils that were phagocytizing bacteria increased from the fitted value of 0.30% to 91.50% ($p = 0.001$). In monocytes it changed from 0.30% to 84.62% ($p = 0.001$). Mean fluorescence also increased significantly in both cases, from 76.31 to 306.10 in neutrophils and from 36.28 to 357.24 (Table 5-2).

Correlations between studied variables: Correlations were assessed between D%PN, D%PM, DFN and DFM and the studied variables. A positive association ($p = 0.041$) was present between the difference in the mean fluorescence (DFM) and the difference in the proportion of phagocytizing (D%M) monocytes. The proportion of neutrophils and monocytes that were phagocytizing was significantly correlated ($p = 0.007$) and the mean fluorescence emitted by monocytes and neutrophils showed weak correlation ($p = 0.071$). There is a positive correlation between total calcium at day 2 and the proportion of phagocytizing neutrophils ($p = 0.017$) and the proportion of phagocytizing monocytes ($p = 0.011$; Table 5-3). Serum total calcium at day 2 was found to be significantly correlated with fecal score on day 2 (Table 5-4). There was also a

correlation between fecal score on day 2 with mean fluorescence of monocytes ($r = 0.702$; $p = 0.016$).

Univariate and multivariate analysis: To construct a model for the D%PN, univariate models were constructed of the variables that showed some correlation with D%PN. These variables were D%PM, serum total calcium at day 2, and fecal score at day 2. Of these, only serum total calcium at day 2 was significant ($p = 0.02$). In the multivariate analysis only serum total calcium at day 2 fitted in the best model ($p = 0.04$).

In the model for D%PM, only serum total calcium at day 2 ($p = 0.02$), which was significant in the univariate analysis, fitted in the multivariate analysis, where DFM, D%PN and fecal score at day 2 where included in the analysis.

The model for DFN initially included serum total calcium at day 2, DFM, and fecal score at day 2, which were the significant variables at day 2. The model forcing serum total calcium at day 2 gave no significant model ($p = 0.30$). The post-hoc power analysis obtained was 0.169.

The multivariate model for DFM included tCa2 ($p = 0.05$), DFN ($p = 0.001$) and D%PM ($p < 0.0001$).

Categorical analyses: Mean tCa2 was 3.38 mmol/L, and this was the cut-off value used to classify normal/high versus low tCa2. Mean values of the flow cytometry variables were used to classify the cellular response as positive or negative as described above (Table 5-5).

Serum total calcium at birth, fecal score at day 2, D%PN and D%PM showed some marginally significant differences between high/normal and low calcium categories (Table 5-6). Fisher's exact test did not show any significant association between tCa2 and any of the flow cytometry variables, but %PN at day 2 had an OR of 12 (95% CI = 0.79 – 180.97) (Table 5-7).

Day 21 of life

Quantification of neutrophils and monocytes: Results of the descriptive analysis of neutrophils and monocytes at day 21 of age were similar to those at day 2, except that SS in monocytes and neutrophils and FS in monocytes, were not significant different in samples with or without bacteria (Table 5-8, Table 5-9).

Correlation between studied variables: Correlations between any of the flow cytometry variables and tCa21 were not found. Mean fluorescence emitted by neutrophils and monocytes were significantly correlated, but other variables did not show significant correlations (Table 5-10).

Univariate and multivariate analyses and categorical analyses: None of the analyses performed showed an association between tCa21 and the flow cytometry variables. Mean tCa21 was 2.85 mmol/L (Table 5-11), and no association was found when the categorization of tCa21 and flow variables was done. None of the variables studied were significantly different between high/normal and low tCa21 groups (Table 5-12).

Cytokines

Only 12 samples on day 2 and 10 on day 21 could be analyzed, due to lost samples. Descriptive variables on day 2 and 21 of life are presented in Tables 5-13 to 5-16, where cytokine data are presented as the values in all sampled calves and in calves with high/normal and low tCa2 (and tCa21). No significant differences are found between means of IFN-gamma and TNF-alpha when comparing calves with high/normal to calves with low tCa (2 or 21).

Correlation analyses did not show any further association between cytokine variables and total calcium on days 2 and 21 (Table 5-17; 5-18). On both sampling days, there was a positive correlation between the IFN-gamma and TNF-alpha production by the calves. No further significant associations were found by categorizing the data.

Discussion

Phagocytic leukocytes, neutrophils and monocytes, were present and functional at both 2 and 21 days of age in the calf. Neutrophils and monocytes were activated when blood was incubated with bacteria for 2 h. This activation produced an increase in the proportion of phagocytizing monocytes and neutrophils and an increase in respiratory burst after bacterial ingestion, measured as mean fluorescence emitted. Similar results have been reported by Menge et al. (1998) where they found that newborn calves have a higher proportion of phagocytizing monocytes and greater mean fluorescence than calves between 3 and 9 weeks of age. In neutrophils they did not find such difference. I found a greater response in calves two days old than in calves at 21 days of age, in both neutrophils and monocytes. Higuchi et al. (1997) also found little difference in the percentage of phagocytosing neutrophils in calves less than 1 week of age compared to calves between 2 and 4 weeks, and reported no difference in intracellular calcium concentration between both age groups. Kampen et al. (2006) reported neutrophil phagocytosis in bovine neonates from the first week of life and with little change during the first six months of life. They also reported a decrease in the burst activity and lack of correlation between phagocytosis and oxidative burst and gamma-globulins, as I found in my study. Immunoglobulins have been previously reported to have opsonic capacity and increased neutrophil phagocytic killing when they are antigen specific and in the presence of complement (Rainard and Boulard, 1992, Rainard et al., 1988). In the study we are presenting here the bacteria used was the k-12 strain of *E. coli*, a non-pathogenic strain against which the cow might not produce antigen specific immunoglobulins, explaining why no correlation was found between IgG concentration and phagocytosis. Menge et al. (1998) found some changes in the percentage of monocytes and neutrophils phagocytizing 4 h after birth in calves deprived of colostrum compared to calves fed colostrum at birth, but the actual immunoglobulin

concentration in those calves were not measured and other immunogenic factors present in colostrum could have had an effect on this.

On day 2 of life, I was able to find a positive correlation between serum total calcium in the calf and the proportion of monocytes and neutrophils that were phagocytizing bacteria. This result needs to be interpreted with caution as I found a positive correlation between serum calcium and fecal score at day 2. No correlation was found between fecal score at day 2 and proportion of phagocytizing cells, but I found a correlation between fecal score and mean fluorescence emitted by monocytes. Therefore, it is possible that calves with higher fecal scores were undergoing a systemic infection, even if no physical signs were present. If that was the case, the inflammatory response would already have started in those calves and a higher response to the added bacteria could be expected. Both monocytes and neutrophils, after being incubated with the bacteria, demonstrated a shift in the FS and SS (increased mean values). I would expected to find this shift if neutrophils and monocytes were naturally stimulated, but when we compared SS and FS of calves with high fecal score compared to calves with fecal score of 1, I found no difference, as I did not find any difference when comparing those values in the groups with high/normal tCa and low tCa.

Intracellular ionic calcium is important in the activation of monocytes and neutrophils (Higuchi et al., 1997, Higuchi et al., 1997, Ortiz-Carranza and Czuprynski, 1992, Zheng et al., 1992). They have described *in vitro* studies the presence of a ionic calcium influx in neutrophils and monocytes following activation. Ortiz-Carranza and Czuprynski (1992) also described that in the absence of extracellular ionized calcium, influx of calcium following cell stimulation does not occur suggesting that extracellular ionic calcium plays an important role in the activation of neutrophils and monocytes. In this study, I tried to find any possible association between serum

total calcium in neonatal calves and the ability of neutrophils and monocytes to be activated after being stimulated with bacteria. I only found a correlation between serum total calcium at day 2 and the proportion of neutrophils and monocytes that were phagocytizing the bacteria. Failure to find other significant associations could be the result of a small sample size, producing a lack of power in the analyses performed, but the OR between the response of neutrophils to phagocyte bacteria with tCa at day 2 of age had a 95% CI of 181 in its upper limit, being possible that calves with higher tCa concentration at day 2 of life would be up to 180 times more likely to have a greater proportion of neutrophils phagocytizing bacteria compared to calves with tCa concentration lower than the mean at day 2. To my knowledge, there are not many studies in the bovine that have investigated the effects of hypocalcemia produces on monocytes or neutrophils. Kimura et al. in 2006 demonstrated that cows suffering clinical hypocalcemia had a decreased calcium influx in neutrophils following activation. This impairment in calcium influx could be one possible factor related to the increased incidence of infectious diseases that cows with hypocalcemia have compared to normocalcemic cows (Curtis et al., 1983, Whiteford and Sheldon, 2005).

To better characterize the role of serum total calcium in monocyte and neutrophil function in the calf, other techniques could be implemented such as a better cell characterization using specific cluster of differentiation (CD) markers for each cell type, use of calcium sensitive dyes to characterize the influx of calcium in neutrophils and monocytes after activation and measurement of ionic calcium in the calf.

Cytokine production by leukocytes is thought to be dependent on calcium influx activation of nuclear factors (Brown et al., 2004, Liu et al., 2008). Blockage of CRAC (calcium release activated calcium) channels reduces cytokine production (Di Sabatino et al., 2009). In my study,

no association between serum total calcium in calves and production of IFN-gamma or TNF-alpha by their leukocytes in response to bacterial stimulation was found.

Conclusion

In conclusion, no definitive associations were found between serum total calcium in the neonatal calf and the quality of its immune response, measured as the ability of neutrophils and monocytes to phagocytize bacteria and produce cytokines in response to a bacterial stimulus. But considering the limitations in sample size, the results obtained with this study are worthwhile to be continued with further investigation, possibly by other methodologies that could focus more in the molecular level.

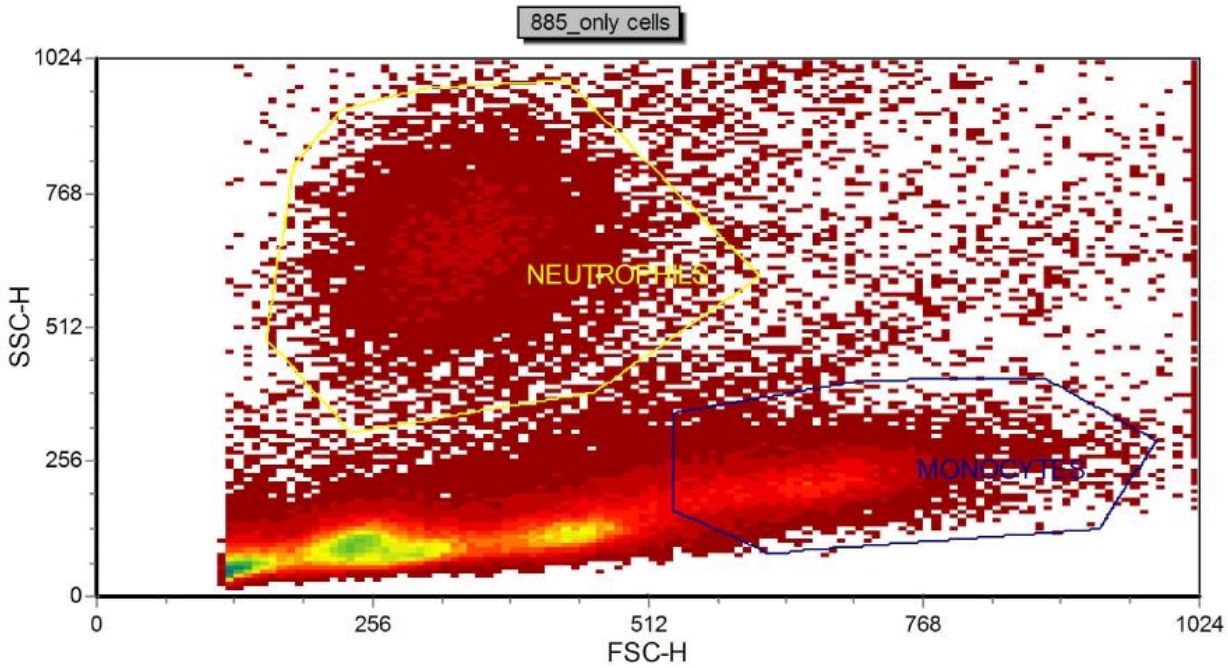

Figure 5-1. Flow cytogram of SS (side scatter) against FS (forward scatter) of blood leukocytes. Monocytes and neutrophils populations are gated based on their size (FS) and granularity (SS).

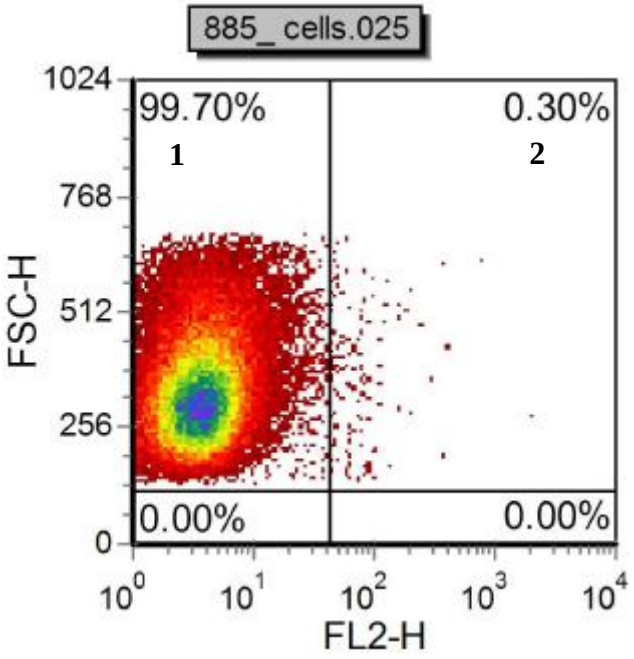

Figure 5-2. Forward scatter versus fluorescence cytogram of gated neutrophils without bacteria. Most of the neutrophils are in region 1, where the emitted fluorescence is low.

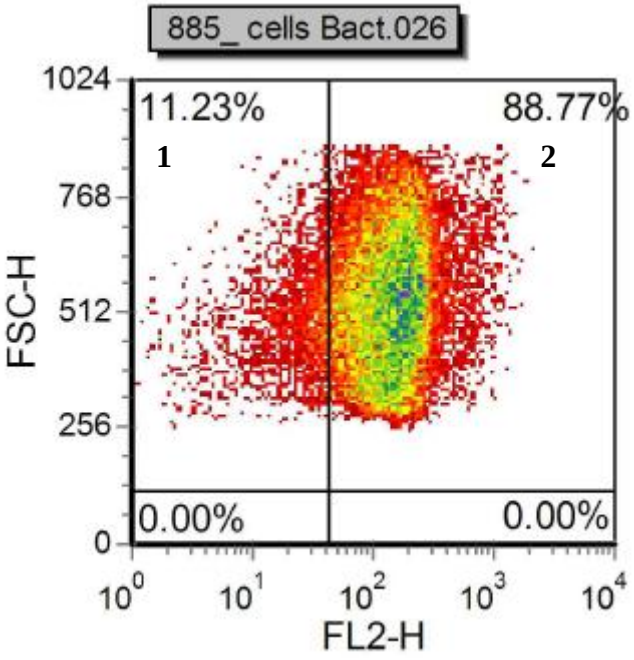

Figure 5-3. Forward scatter versus fluorescence cytogram of gated neutrophils with bacteria. There has been a shift of the neutrophil population towards region 2, where the emitted fluorescence by neutrophils is greater than in region 1.

Table 5-1. Descriptive analysis of flow cytometer SS vs FS on blood from calves at day 2 of age.

Group			Control				Bacteria			
Variable	p-value	N	Mean	Min	Max	SE	Mean	Min	Max	SE
%Neutrophils	0.011	13	39.05	24.04	61.88	3.41	23.72	8.46	49.0	3.23
%Monocytes	0.001	13	8.54	4.77	13.24	0.68	4.21	1.74	6.20	0.36
FS Neutrophils	0.001	13	375.34	334.16	423.99	8.71	515.09	438.87	582.54	11.63
SS Neutrophils	0.039	13	514.30	437.83	553.76	9.19	558.60	447.94	679.85	18.16
FS Monocytes	0.005	13	661.57	630.29	689.58	4.98	688.01	667.76	725.24	4.01
SS Monocytes	0.028	13	211.73	191.98	257.30	4.56	232.31	205.61	287.01	6.73

FS = Forward scatter. SS = Side scatter.

Table 5-2. Descriptive analysis of forward scatter vs emitted fluorescence on blood from calves at day 2 of age.

Group			Control				Bacteria			
Variable	p-value	N	Mean	Min	Max	SE	Mean	Min	Max	SE
% PN	0.001	13	0.31	0.27	0.33	0.005	91.50	78.43	99.64	1.63
MFN	0.001	13	76.31	34.91	102.04	5.52	306.10	173.86	418.31	19.73
% PM	0.001	13	0.3	0.25	0.35	0.009	84.62	67.59	95.87	2.55
MFM	0.001	13	36.28	17.41	80.88	4.69	357.24	132.10	598.81	34.57

%PN = Percentage phagocytizing neutrophils.
MFN = Mean fluorescence emitted by neutrophils.
%PM = Percentage phagocytizing monocytes.
MFM = Mean fluorescence emitted by monocytes.

Table 5-3. Spearman's correlation matrix and accompanying p-values between flow cytometry variables and serum total calcium at 2 days.

	Statistic	D%PN	DFN	D%PM	DFM
DFN	r	0.302	1		
	p	0.316			
D%PM	r	0.709	-0.033	1	
	p	0.007	0.915		
DFM	r	0.352	0.516	0.571	1
	p	0.239	0.071	0.041	
tCa2	r	0.646	0.273	0.679	0.337
	p	0.017	0.367	0.011	0.261
IgG	r	0.149	0.075	0.244	0.119
	p	0.625	0.807	0.421	0.699

D%PN = Difference in the percentage phagocytizing neutrophils between controls and samples stimulated with bacteria.
D%PM = Difference in the percentage phagocytizing monocytes between controls and samples stimulated with bacteria.
DFN = Difference in the mean fluorescence emitted by neutrophils between controls and samples stimulated with bacteria.
DFM = Difference in the mean fluorescence emitted by monocytes between controls and samples stimulated with bacteria.
tCa2 = Serum total calcium at 2 days.
IgG = Immunoglobulin G measured by SRID.

Table 5-4. Spearman's correlation matrix and accompanying p-values between serum total calcium, fecal score and albumin.

	Statistic	tCa0	tCa2	Fecal Score 2
tCa2	r	0.533		
	p	0.061		
Fecal Score 2	r	0.497	0.639	
	p	0.120	0.034	
Albumin 2	r	0.384	0.198	0.578
	p	0.195	0.517	0.062

tCa_n = serum total calcium on sample day.
Fecal score 2 = fecal score at 2 days of age.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-5. Flow cytometry variables in all calves at 2 days of age.

Variable	N	Mean	Minimum	Maximum	SE
tCa0 (mmol/L)	13	3.38	3.18	3.72	0.05
tCa2 (mmol/L)	13	3.38	3.12	3.70	0.06
IgG (mg/dL)	13	1574	653	3170	187.86
Age Sick (days)	5	24	7	34	4.83
D%PN	13	91.19	78.1	99.34	1.67
D%PM	13	84.32	67.29	95.56	2.56
DFN	13	229.79	71.82	340.9	21.33
DFM	13	320.97	90.82	576.78	34.06
Fecal Score 2	11	1.8	1	4	0.38

tCa_n = serum total calcium on sample day.
IgG = immunoglobulin G at 2 days.
D%PN = Difference in the percentage phagocytizing neutrophils between controls and samples stimulated with bacteria.
D%PM = Difference in the percentage phagocytizing monocytes between controls and samples stimulated with bacteria.
DFN = Difference in the mean fluorescence emitted by neutrophils between controls and samples stimulated with bacteria.
DFM = Difference in the mean fluorescence emitted by monocytes between controls and samples stimulated with bacteria.
Age Sick = age at diagnosis.
Fecal score 2 = fecal score at 2 days of age.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-6. Flow cytometry variables in calves classified by serum total calcium at 2 days of age.

Group	High tCa2			Low tCa2			
Variable	N	Mean	SE	N	Mean	SE	p-value
tCa0 (mmol/L)	7	3.50	0.06	6	3.25	0.03	0.020
tCa2 (mmol/L)	7	3.55	0.04	6	3.18	0.01	0.005
IgG (mg/dL)	7	1828	283.96	6	1277	194.61	0.109
Age Sick (days)	2	19	11.50	3	28	4.10	0.297
D%PN	7	93.81	1.09	6	88.14	3.08	0.054
D%PM	7	88.01	2.35	6	80.01	4.40	0.071
DFN	7	254.05	26.27	6	201.49	33.21	0.168
DFM	7	370.44	44.27	6	263.25	45.29	0.138
Fecal Score 2	6	2.5	0.56	5	1	0	0.030

tCa_n = serum total calcium on sample day.
IgG = immunoglobulin G at 2 days.
D%PN = Difference in the percentage phagocytizing neutrophils.
D%PM = Difference in the percentage phagocytizing monocytes.
DFN = Difference in the mean fluorescence emitted by neutrophils.
DFM = Difference in the mean fluorescence emitted by monocytes.
Age Sick = age at diagnosis.
Fecal Score 2 = fecal score at 2 days of age.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-7. Contingency tables and Fisher tests for association between flow variables and classification of serum total calcium on calves day 2.

%PN2	RESPONSE	
	Positive	Negative
High tCa2	6	1
Low tCa2	2	4

MFN2	RESPONSE	
	Positive	Negative
High tCa2	5	2
Low tCa2	2	4

%PM2	RESPONSE	
	Positive	Negative
High tCa2	5	2
Low tCa2	2	4

MFM2	RESPONSE	
	Positive	Negative
High tCa2	3	4
Low tCa2	3	3

High tCa2 = calves with serum total calcium greater or equal to the mean of serum total calcium of calves in the study population.
Low tCa2 = calves with serum total calcium lower than the mean of serum total calcium of calves in the study population.

Variable	OR	p-value	95%CI	
%PN2	12	0.086	0.790	180.97
MFN2	5	0.208	0.472	52.96
%PM2	5	0.208	0.472	52.96
MFM2	0.75	0.791	0.080	6.71

%PN2 = Percentage phagocytizing neutrophils at 2 days.
%PM2 = Percentage phagocytizing monocytes at 2 days.
MFN2 = Mean fluorescence emitted by neutrophils at 2 days.
MFM2 = Mean fluorescence emitted by monocytes at 2 days.

Table 5-8. Descriptive analysis of flow cytometer SS vs FS on blood from calves at day 21 of age.

Group			Control				Bacteria			
Variable	p-value	N	Mean	Min	Max	SE	Mean	Min	Max	SE
%Neutrophils	0.001	14	18.82	10.07	34.30	1.75	8.51	2.83	12.94	0.73
%Monocytes	0.002	14	9.18	1.28	16.97	1.14	5.12	3.26	10.22	0.53
FS Neutrophils	0.001	14	361.08	291.38	532.31	19.95	494.55	442.46	584.82	10.95
SS Neutrophils	0.177	14	556.54	482.95	652.96	12.46	593.23	515.41	797.66	24.17
FS Monocytes	0.158	14	693.84	651.48	800.34	10.52	707.39	666.85	749.90	6.42
SS Monocytes	0.397	14	215.70	186.67	248.14	4.08	221.25	177.14	270.63	5.94

FS = Forward scatter. SS = Side scatter.

Table 5-9. Descriptive analysis of forward scatter vs emitted fluorescence on blood from calves at day 21 of age.

Group			Control				Bacteria			
Variable	p-value	N	Mean	Min	Max	SE	Mean	Min	Max	SE
% PN	<0.0001	14	0.30	0.28	0.32	0.004	80.68	47.85	98.21	3.94
MFN	0.0003	14	124.53	30.07	507.00	31.14	247.92	103.60	331.86	16.90
% PM	<0.0001	14	0.32	0.27	0.39	0.01	61.96	48.03	87.73	3.40
MFM	<0.0001	14	37.35	16.41	129.09	8.76	275.37	175.34	368.07	12.88

%PN = Percentage phagocytizing neutrophils.
MFM = Mean fluorescence emitted by monocytes..
%PM = Percentage phagocytizing monocytes.
MFN = Mean fluorescence emitted by neutrophils.

Table 5-10. Spearman's correlation matrix and accompanying p-values between flow cytometry variables and total calcium on day 21 and IgG at 2 days.

	Statistic	D%PN	DFN	D%PM	DFM
DFN	r	-0.169			
	p	0.563			
D%PM	r	0.516	0.077		
	p	0.059	0.794		
DFM	r	-0.178	0.789	0.152	
	p	0.543	0.001	0.605	
IgG	r	-0.008	0.072	0.395	0.092
	p	0.977	0.806	0.162	0.755
tCa21	r	-0.251	-0.004	0.011	0.075
	p	0.387	0.988	0.970	0.799

D%PN = Difference in the percentage phagocytizing neutrophils.
DFN = Difference in the mean fluorescence emitted by neutrophils.
D%PM = Difference in the percentage phagocytizing monocytes.
DFM = Difference in the mean fluorescence emitted by monocytes.
tCa21 = Serum total calcium at 21 days.
IgG = Immunoglobulin G measured by SRID at 2 days.

Table 5-11. Flow cytometry variables in all calves at 21 days of age.

Variable	N	Mean	Minimum	Maximum	Std Dev	Std Error
tCa0 (mmol/L)	14	3.35	3.18	3.37	0.18	0.05
tCa21 (mmol/L)	14	2.85	2.50	3.40	0.30	0.08
IgG (mg/dL)	14	1574	653	3170	700.07	187.10
AgeSick (days)	6	25	15	34	7.25	2.96
D%PN	14	80.38	47.57	97.92	14.74	3.94
D%PM	14	61.64	47.71	87.41	12.72	3.40
DFN	14	123.39	-204.80	258.75	111.80	29.88
DFM	14	238.02	158.93	344.53	50.18	13.41
Fecal Score 21	14	2	1.7	2.8	0.30	0.08

tCa_n = serum total calcium on sample day.
IgG = immunoglobulin G at 2 days.
D%PN = Difference in the percentage phagocytizing neutrophils between controls and samples stimulated with bacteria.
D%PM = Difference in the percentage phagocytizing monocytes between controls and samples stimulated with bacteria.
DFN = Difference in the mean fluorescence emitted by neutrophils between controls and samples stimulated with bacteria.
DFM = Difference in the mean fluorescence emitted by monocytes between controls and samples stimulated with bacteria.
Fecal Score 21 = mean of fecal scores from day 1 to day 21.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-12. Flow cytometry variables in calves classified by serum total calcium at 21 days of age.

Group	High tCa21			Low tCa21			
Variable	N	Mean	Std Error	N	Mean	Std Error	p-value
tCa0 (mmol/L)	7	3.40	0.08	7	3.30	0.06	0.209
tCa21 (mmol/L)	7	3.08	0.08	7	2.60	0.04	0.004
IgG (mg/dL)	7	1534	188.70	7	1614	339.95	0.500
AgeSick (days)	1	34	.	5	23	2.85	0.102
D%PN	7	79.78	5.08	7	80.98	6.43	0.377
D%PM	7	62.43	5.64	7	60.84	4.24	0.425
DFN	7	149.98	25.62	7	96.80	54.56	0.287
DFM	7	240.40	14.04	7	235.64	24.09	0.377
Fecal Score 21	7	2.0	0.07	7	2.1	0.15	0.475

tCa_n = serum total calcium on sample day.
IgG = immunoglobulin G at 2 days.
D%PN = Difference in the percentage phagocytizing neutrophils between controls and samples stimulated with bacteria.
D%PM = Difference in the percentage phagocytizing monocytes between controls and samples stimulated with bacteria.
DFN = Difference in the mean fluorescence emitted by neutrophils between controls and samples stimulated with bacteria.
DFM = Difference in the mean fluorescence emitted by monocytes between controls and samples stimulated with bacteria.
Fecal Score 21 = mean of fecal scores from day 1 to day 21.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-13. Studied cytokine variables in calves at 2 days of age.

Variable	N	Mean	Std Error
S/P CONTROL TNF	12	0.79[a]	0.06
S/P CONA TNF	12	0.89[a]	0.07
S/P Difference TNF	12	0.10	0.05
IFN CONTROL (pg/mL)	12	13.53[a]	3.37
IFN CONA (pg/mL)	12	43.95[b]	16.22
IFN Difference (pg/mL)	12	30.42	14.66
tCa0 (mmol/L)	12	3.40	0.05
tCa2 (mmol/L)	12	3.42	0.06
MEAN FECAL	12	1.5	0.17
AgeSick (days)	4	19.5	4.73
IgG (mg/dL)	12	1711	193.56

Significance $p < 0.05$ with different superscript. Same superscript is no significant.
S/P = sample to positive ratio
S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
Mean fecal = mean fecal score at 2 days.
tCa_n = serum total calcium on sample day
IgG = immunoglobulin G at 2 days.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-14. Studied cytokine variables by classified serum total calcium in calves at 2 days of age.

Group	High tCa2			Low tCa2			
Variable	N	Mean	Std Error	N	Mean	Std Error	p-value
S/P CONTROL TNF	7	0.73	0.06	5	0.88	0.11	0.11
S/P CONA TNF	7	0.86	0.10	5	0.94	0.09	0.28
S/P Difference TNF	7	0.12	0.08	5	0.06	0.04	0.47
IFN CONTROL (pg/mL)	7	14.33	3.84	5	12.41	6.59	0.34
IFN CONA (pg/mL)	7	31.49	8.22	5	61.39	38.26	0.47
IFN Difference (pg/mL)	7	17.16	8.20	5	48.98	33.61	0.19
tCa0 (mmol/L)	7	3.45	0.07	5	3.30	0.07	0.07
MEANFECAL	7	1.5	0.22	5	1.4	0.29	0.33
AgeSick (days)	2	14	7.00	2	25	5.00	0.22
IgG (mg/dL)	7	1870	286.93	5	1489	230.51	0.26

S/P = sample to positive ratio
S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
tCa_n = serum total calcium on sample day
Mean fecal = mean fecal score at 2 days.
IgG = immunoglobulin G at 2 days.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).
High tCa2 = calves with serum total calcium greater or equal to the mean of serum total calcium of calves in the study population.
Low tCa2 = calves with serum total calcium lower than the mean of serum total calcium of calves in the study population.

Table 5-15. Studied cytokine variables in calves at 21 days of age.

Variable	N	Mean	Std Error
S/P CONTROL TNF	10	0.57[a]	0.01
S/P CONA TNF	10	0.79[b]	0.11
S/P Difference TNF	10	0.22	0.11
IFN CONTROL (pg/mL)	10	9.71[a]	1.79
IFN CONA (pg/mL)	10	271.15[b]	75.18
IFN Difference (pg/mL)	10	261.44	75.87
tCa0 (mmol/L)	10	3.37	0.06
tCa21 (mml/L)	10	2.82	0.11
MEAN FECAL	10	2.1	0.11
Age Sick (days)	6	25	2.96
IgG (mg/dL)	10	1355	167.60

Significance $p < 0.05$ with different superscript. Same superscript is no significant.
S/P = sample to positive ratio
S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
tCa_n = serum total calcium on sample day
Mean fecal = mean fecal score during the first 21 days of age.
IgG = immunoglobulin G at 2days.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-16. Studied cytokine variables in calves at 21 days of age, by serum total calcium classified as low or high.

Group	High tCa21			Low tCa21			
Variable	N	Mean	Std Error	N	Mean	Std Error	p-value
S/P CONTROL TNF	4	0.57	0.03	6	0.56	0.01	0.46
S/P CONA TNF	4	0.61	0.06	6	0.91	0.17	0.14
S/P Difference TNF	4	0.04	0.04	6	0.34	0.18	0.08
IFN CONTROL (pg/mL)	4	8.6	2.15	6	10.45	2.73	0.24
IFN CONA (pg/mL)	4	257.74	145.76	6	280.09	91.76	0.46
IFN Difference (pg/mL)	4	249.15	146.79	6	269.64	92.83	0.46
tCa0 (mmol/L)	4	13.9	0.49	6	13.2	0.25	0.16
MEAN FECAL	4	2	0.1	6	2.1	0.17	0.46
Age Sick (days)	1	34	.	5	23	2.85	0.16
IgG (mg/dL)	4	1355	204.27	6	1354	259.95	0.49

S/P = sample to positive ratio
S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
Mean fecal = mean fecal score during the first 21 days of age.
IgG = immunoglobulin G at 2 days.
High tCa21 = calves with serum total calcium greater or equal to the mean of serum total calcium of calves in the study population.
Low tCa21 = calves with serum total calcium lower than the mean of serum total calcium of calves in the study population.
Fecal score: 0 = normal, well formed feces; 1 = pasty, softer than normal feces; 2 = mild diarrhea, semi-liquid with a solid component; 3 = pure liquid feces (Walker et al. 1998).

Table 5-17. Pearson's correlation matrix and accompanying p-values at 2 days.

	Statistic	S/P Difference TNF	IFN Difference
	r	0.720	
IFN Difference	p	0.008	
	r	0.004	-0.253
tCa2	p	0.991	0.428

S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
tCa2 = serum total calcium at 2 days.

Table 5-18. Pearson's correlation matrix and accompanying p-values at 21 days.

	Statistic	S/P Difference TNF	IFN Difference
	r	0.648	
IFN Difference	p	0.043	
	r	-0.109	0.207
tCa21	p	0.764	0.567

S/P difference = S/P conA – S/P control
IFN difference = IFN conA – IFN control
tCa21 = serum total calcium at 21 days.

CHAPTER 6
CONCLUSION

Serum calcium concentration in calves has been widely investigated, but the results obtained in the present thesis are novel. We reported a possible association between low serum total calcium in the calf with an increase probability of being diagnosed with respiratory infection and otitis media. The exact causes of why these calves may present lower concentration of calcium in blood are not known. Possible causes include reduced dietary calcium intake, hormonal dysfunction or increased calcium excretion.

The effect of serum total calcium concentration on immune cell function and cytokine production reported in this study are also important. The limitations presented in this study with the small sample size and the variation between calves gave results that need to be interpreted with caution. Some important associations were found in neutrophil phagocytosis and calcium concentration on day 2 of age, and further studies to investigate this association are encouraged.

The calcium levels reported in this study were no where near those that produce clinical signs of hypocalcemia. In cows with clinical hypocalcemia, there is a reduced calcium influx in their peripheral blood mononuclear cells. The management, animal care and nutrition provided to the calves in the farm where the study was conducted were of excellent quality. The effect that improper nutrition to calves could produce in the concentration of serum total calcium, and on their immune function is not known.

Perhaps a better way to asses the importance of calcium concentration in calves' immune function would be to use molecular technology. Intracellular calcium concentration can be measured, and its correlation to calcium concentration in blood and in the ER could be studied, therefore obtaining a better picture of the calcium concentration in the whole calf. The concentration of calcium in the different compartments in the calf and their effects on the

immune system could be investigated. One could also study the effect of experimentally reduced levels of calcium on immune function, but ethical considerations would have to be taken in account in this case.

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BIOGRAPHICAL SKETCH

Beatriz Sanz Bernardo was born in 1982, in Madrid, a city located in Spain, full of history and nice places. Her family was from northern Spain, Segovia and Valladolid, cities considered as her second home. She grew up in a city where not much contact with animals, but she spend all summers in her childhood in smaller towns, where she contacted nature and began loving it.

School days passed surrounded by good friend and she finally decided to study veterinary sciences at the Universidad Complutense de Madrid, where she initiated her interest for ruminant medicine. After fulfilling her studies she decided to leave Spain to know new places and different cultures, beginning working at UK, and living in Aberdeen, Scotland for six month while she was working for the Meat Hygiene Service of UK. She shortly realized that although enjoying her job she missed the clinical side of the veterinary work and decided to apply for an internship in USA, being hired at the University of Florida.

Internship year passed very quickly and she still wanted to be exposed to more challenges, and she was also tented to the possibility of widening her knowledge by enrolling in a Master program. The research environment in which she was involved was very impressive to her and she wanted to be part of it.

Now, she is ready to go to practice but she will never forget the time she spend in Florida and the friends she is leaving there. She does not know yet what will be the next step in her life, but research and immunology has gotten deep within her, so the doors to a PhD will never get closed.