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Influence of Vitamin E on Innate and Humoral Components of the Immune System of Pregnant Ewes and Their Lambs

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INFLUENCE OF VITAMIN E ON INNATE AND HUMORAL COMPONENTS OF THE IMMUNE SYSTEM OF PREGNANT EWES AND THEIR LAMBS

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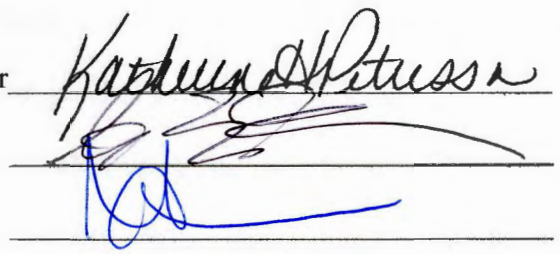
Major Professor 

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ABSTRACT

The effect of parenteral administration of vitamin E during the pregnancy and lactation period of ewes and their lambs on innate and humoral components of the immune system was investigated in a 3 part study. 1.) Pregnant/Lactating ewes: Fifteen pregnant Dorset sheep were supplemented with vitamin E (30 IU d-α-tocopherol /Kg body weight (BW), E, n=10) or placebo (emulsified base, P, n=5) from the last trimester of gestation through weaning. Blood and colostrum were analyzed every 2 wk for α-tocopherol, selenium, glutathione peroxidase activity, lysozyme activity, total IgG and IgG antibodies against tetanus toxoid. 2.) Lambs (0-8wks): Lambs born to E and P supplemented ewes received either vitamin E (30 IU d-α-tocopherol /Kg BW) or placebo (emulsified base) every 2 weeks from birth through 8 wk of age. This resulted in four treatment groups: E/E (n=10), E/P (n=11), P/E (n=4), P/P (n=5). The lambs were sampled every other week and their blood was analyzed as for the ewes. 3.) Lambs (Vaccination period): When the youngest lamb reached 8 wks of age all lambs were vaccinated as a group against *Clostridium perfringens* and *Clostridium tetani*. Booster vaccinations were given 4 wk later. Blood was sampled prior to primary vaccination, 1 wk post vaccination and then every 2 wk until 7 wk post vaccination. Results: Pregnant/Lactating ewes: Serum vitamin E concentrations increased over time in supplemented ewes as compared to placebo ewes. Colostrum vitamin E levels were also increased in supplemented versus placebo ewes. Vitamin E supplementation did not influence plasma selenium, glutathione peroxidase activity and serum lysozyme, IgG and IgG against tetanus toxoid in the serum or colostrum of ewes. There were fluctuations within treatment in the production of IgG in response

to parturition. Lambs (0-8wks): Lambs directly supplemented with vitamin E in addition to maternal supplementation (E/E) had higher levels of serum α-tocopherol than the placebo group (P/P). There were no differences in plasma selenium, glutathione peroxidase activity, serum lysozyme, IgG and IgG against tetanus toxoid concentrations between groups. There was a loss of maternal antibodies over time in all lambs. Serum lysozyme activity was higher at birth, prior to ingestion of colostrum, in lambs that received maternal vitamin E supplementation compared to the lambs that received only direct vitamin E supplementation. Lambs (Vaccination period): Serum α-tocopherol levels increased in lambs that received direct vitamin E supplementation (E/E and P/E) irrespective of maternal supplementation (P/P and E/P). Plasma selenium, glutathione peroxidase activity, serum lysozyme and IgG did not differ between treatment groups. There was an increase in the production of IgG against tetanus toxoid in response to booster vaccination in PE lambs. Conclusions: Supplemental vitamin E to the ewes and lambs, at 30 IU/Kg BW, had a modest effect on parameters used in this study to assess innate and humoral immunity.

(**Key words:** vitamin E, ewe, IgG, tetanus toxoid)

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DEDICATION

To my Parents

for their endless support and love

PREFACE

This thesis is written in a manuscript format. Chapter 1 includes detailed information on current vitamin E research. A condensed paper about the study is presented in chapter 2. Additional details on methods used throughout the study are presented in the appendices.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1:

Review of Literature

INTRODUCTION

The immune system protects the host from invading microorganisms through complex pathways of recognition and elimination. Disease occurs when the immune system is compromised by infectious pathogens. Humans and animals are susceptible to infections throughout their lives, but are more susceptible when undergoing physiological stress, malnourishment or at varying stages of life such as birth, gestation and lactation. Infections are the major cause of production losses and can affect animals of all ages. The nutritional status of an animal can influence host resistance and susceptibility to disease (Hidiroglou, et al., 1992). Effects of nutrition on disease resistance have long been recognized and the knowledge is being applied to farm animal production systems and particularly to ruminants. Nutritional supplementation to improve host immunity has always been an area of interest to researchers and is the subject of this thesis.

There are 5.7 million sheep and lambs in United States (USDA, 2008). These sheep are used for their meat, wool, milk, hide, and other by products. Losses to the sheep industry from nonpredator causes account for $33 million annually (USDA, 2005) with the majority of these losses attributed to infections. About 15-50% mortality is seen before weaning, with the most of the mortality occurring in first 24 hrs of life (Bekele, et al., 1992, Rowland, et al., 1992). This increased susceptibility can be attributed to immature immune system of the neonates.

Supplementation with vitamin E has been shown to modulate immune function in humans (Meydani, et al., 1990) and several species of animals including ruminants (Afzal, et al., 1984, Beharka, et al., 1997, Ellis and Vorhies, 1976, Moriguchi and Muraga, 2000, Reddy, et al., 1987, Ritacco, et al., 1986, Segagni, 1955, Tengerdy, 1989). Although vitamin E has been investigated extensively, little research has been undertaken to establish whether supplementation of vitamin E to small ruminants during gestation, lactation and early in life has a beneficial impact on immune function in these animals.

Innate Immunity

In general, the immune system can be broadly categorized into two divisions: innate and adaptive. The cells of the innate immune system serve as the first line of defense and act nonspecifically towards antigens. The innate response is mediated through antimicrobial molecules such as lysozyme, lactoferrin, nitric oxide, and the various cells of defense i.e. natural killer cells, macrophages, neutrophils, and mast cells.

Lysozyme is a hydrolytic enzyme present in serum, tears, seminal fluid, milk, and in phagocytic cells. It has bactericidal effect on most gram-positive and some gram-negative bacteria and viruses. It binds to the surface of the susceptible pathogens and promotes their lysis (Tizard, 2000).

Glutathione peroxidase (GSH-Px) is an enzymatic antioxidant that belongs to the selenoprotein family and catalyzes the reduction of hydrogen and lipid peroxides to their corresponding alcohols (Loscalzo, 2008). It plays a key role in maintaining

the physiological balance between prooxidants and antioxidants (Zablocka and Janusz, 2008).

Adaptive Immunity

The adaptive immune response constitutes humoral and cell mediated immunities that use B and T lymphocytes respectively to neutralize pathogens in an antigen specific manner. Humoral immunity is mediated through antibodies whereas cell mediated immunity is mediated through cytokines, namely interleukins and interferons, that act as chemical messengers (Tizard, 2000). B-lymphocytes that mature in bone marrow provide humoral immunity through antibodies - IgG, IgM, IgA, IgD, and IgE. Antibodies bind to specific antigens and enhance their destruction and elimination. Blood contains high levels of IgG followed by IgM and then IgA. T lymphocytes mature in thymus and are divided into T helper and T cytotoxic cells to lyse extracellular and intracellular organisms. Cytokines produced by the cellular immune system act as chemical messengers further activating B and T lymphocytes as well as other immune cells such as natural killer cells and macrophages (Tizard, 2000).

Maternal immune system

The maternal immune system during the transition period, which includes pregnancy, parturition, and lactation, is under extreme stress. The stress during pregnancy and increased metabolic demands during parturition and lactation result in increased production of reactive oxygen species leading to oxidative stress (Spears and Weiss, 2008). In addition to these drains on the animal's physical resources, they must be in a suitable physiological state to ensure repair of any tissue damaged during

parturition, and to maintain resistance to infections (Andrieu, 2008). This physiological stress can have a negative impact on neutrophil function, antibody responses, and cytokine production by immune cells (Spears and Weiss, 2008). The compromised immune system of the dam not only influences their own immune function but can also affect their offspring.

Neonatal immune system

Neonates have an immature immune system that renders them susceptible to a wide variety of bacterial and viral pathogens. They have impaired immune responses due to a range of deficiencies in both adaptive and innate immunity. Additionally, maternal antibodies transferred with colostrum activate and regulate innate responses present in the neonate to fight against infection, but at the same time can interfere with the neonates ability to develop specific immunity against various antigens (Chase, et al., 2008). The interaction of the maternal and neonatal immune system is depicted in Fig. 1. Immunity is low at birth and a peak in passive immunity occurs after ingestion of colostrum. Passive immunity offers protection to the newborn through maternal antibodies. Active immunity, which has to be developed by the newborn, is low at birth and gradually increases with age. When passive immunity starts to wane and active immunity is not fully established to confer sufficient protection there is a window of susceptibility to infections (Chase, et al., 2008).

Infections are a major cause of neonatal losses. Newborns can acquire infections either from the environment or from the dam. Additionally, maternal hormones produced prior to parturition can lead to immunosuppression in the offspring (Jacob, et al., 2001). Overall, these infections, coupled with a naïve immune

system, leads to significant losses either from mortality or decreased production efficiency. To protect newborns from infectious pathogens and increase their disease resistance, the immune function of the dam, newborn or both needs to be enhanced.

As the epitheliochorial placenta of ruminants does not allow passage of immunoglobulins from dam to fetus, colostrum is the only efficient source of immune components and nutrients to the neonate. Colostrum contains proteins, immunoglobulins, nonprotein nitrogen, fat, vitamins and minerals. Hence, improving the dam's immune system may increase immunoglobulin transfer to the newborns through the colostrum. Boosting the newborns own immune system will increase their resistance to infections. Any measures that can be shown to improve host resistance to infections will have a major impact in reducing morbidity, mortality, and improving production efficiency.

REACTIVE OXYGEN SPECIES AND ANTIOXIDANTS

The maternal immune system during gestation and lactation and neonatal immune system is under extreme physiological stress. During these periods, there can be increased production of reactive oxygen species which can be harmful to the host.

In aerobic organisms, oxygen is reduced to water, the end product of mitochondrial respiration. During this process, there is leakage of single electrons leading to the partial reduction of O_2 to superoxide anion ($O_2^{\cdot -}$) (Frei, 1994). These free radicals (reactive oxygen, nitrogen and chlorine species) due to their unpaired electrons are highly unstable and reactive. In addition to the electron transport, there are various endogenous pathways for the production of reactive oxygen species (ROS): Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase in

phagocytic cells (neutrophils and monocytes), xanthine oxidase, epinephrine, and quinoid substrates, such as coenzyme Q10 and the cytochrome P450 system (Frei, 1994).

Beneficial aspects of ROS

Recent work on reactive oxygen species has revealed their beneficial aspects to the host in disease prevention by assisting the immune system in cell signaling and playing an essential role in apoptosis (Seifried, et al., 2007). Innate immune response, characterized by the production of ROS within the phagocytic cells such as neutrophils and macrophages is a crucial event in the phagocytosis and subsequent destruction of the invading pathogen (Bogdan, et al., 2000, Droge, 2002). During the respiratory burst, high levels of superoxide anions and hydrogen peroxide are produced by NADPH oxidase which is also a part of innate defense (Segal, et al., 2000). Hence, any activities that can block the production of ROS may result in increased bacterial or fungal infections and increased mortality (DeForge, et al., 1993, Segal, et al., 2000).

Deleterious aspects of ROS

The ROS formed during cellular processes may damage various biological macromolecules, which includes damage to cellular membranes and lipoproteins by lipid peroxidation, damage to proteins leading to structural changes, altered enzyme activity and oxidative damage to DNA (Frei, 1994).

Antioxidants protect biological macromolecules from harmful effects of free radicals either by scavenging the free radicals or by stopping free radical propagation (Frei, 1994). The antioxidant defense can be classified into enzymatic and

nonenzymatic defenses. Enzymatic includes Glutathione peroxidase (GsHPx) , catalase and superoxide dismutase (SOD) whereas nonenzymatic includes vitamin E, vitamin C, β-carotene and others (Evans and Halliwell, 2001).

VITAMIN E

Vitamin E is a lipid soluble vitamin discovered in 1922 by Evans and Bishop (Evans and Bishop, 1922). The active substance was isolated by Evans in 1936 and was named as tocopherol which means "to bear offspring" as it was shown to prevent sterility in rats (Emerson, et al., 1936). Since then, there has been extensive research directed towards the mechanism and role of this multifunctional vitamin.

Structure of vitamin E

Vitamin E was first found in wheat germ oil and lettuce (Wang and Quinn, 1999). Vitamin E is also present in many plants, legume and fruit sources in nature (NIH, 2009). Vitamin E occurs as eight naturally occurring compounds called tocopherols and tocotrienols which differ in their biological activity (IUPAC-IUB, 1982) and the saturation of phytal side chain (Fig. 2). Different isoforms of vitamin E include α-, β-, γ-, δ-tocopherols and α-, β-, γ-, δ-tocotrienols (Kayden and Traber, 1993). Within each group α-, β-, γ-, δ are designated depending on the position and number of methyl groups on the aromatic ring. Among all the forms of vitamin E, α-tocopherol has the highest biological activity attributed to a higher number of methyl species that can scavenge the peroxyl radicals (Meydani, 1995).

Metabolism of vitamin E

As a fat soluble vitamin, the metabolism of vitamin E is closely related to other lipids and lipoproteins. In the small intestine, vitamin E is integrated into micelles

which are composed of products of lipid hydrolysis, biliary secretions and dietary lipids (Rimbach, et al., 2002). Micelle formation facilitates the absorption of vitamin E and the action of pancreatic lipases which further aid in the absorption of vitamin E into the enterocytes lining the intestinal lumen (Brigelius-Flohe and Traber, 1999). Vitamin E is then packaged in the enterocytes along with other lipids, as chylomicrons or chylomicron remnants, which are absorbed and carried by lymphatic circulation to the liver (Bjorneboe, et al., 1987, Kayden and Traber, 1993, Traber, et al., 1986).

In the liver α- tocopherol is secreted into plasma in conjunction with very low density lipoprotein (VLDL) (Cohn, et al., 1988). Alpha tocopherol associated with VLDL in plasma can have different fates, it can either be transferred to LDL during VLDL catabolism, get delivered to HDL during lipolysis or get transferred back to the liver via VLDL remnants (Kayden and Traber, 1993). Liver regulates the secretion and excretion of different forms of tocopherol. The other forms of ingested tocopherol, other than alpha tocopherol undergoe rapid clearance via bile (Kayden and Traber, 1993). This preferential secretion of alpha tocopherol by liver is due to α-tocopherol transfer protein (α-TTP) as observed in knockout mice experiments (Leonard, et al., 2002) and patients who were deficient in α-TTP (Traber, et al., 1990) The major route of excretion for vitamin E is through the feces. Urinary excretion of vitamin E in the form of carboxyethyl-hydroxychromane (CEHC) metabolites also takes place in case of excess vitamin E intake (Brigelius-Flohe and Traber, 1999).

Mechanism of action of vitamin E

Vitamin E is widely known for its effective chain breaking antioxidant function (Azzi, 2007). Vitamin E is present in all biological cell membranes and

offers protection to the cell membrane lipids from peroxidation and maintains their structural integrity. Cellular integrity is very important for receiving and responding to the messages needed to produce an effective immune response (Latshaw, 1991). Although, vitamin E content varies throughout the body, it is present in highest concentrations in tissues with high lipid content (Combs, 1991) (e.g., liver and adipose tissue). It is also known for scavenging the free radicals produced during normal cellular metabolism and during various immune mechanisms like phagocytosis and respiratory burst (Hogan, et al., 1993a).

Vitamin E (α - TOH) works as an antioxidant by donating a hydrogen group to the unstable free radicals (ROO-) forming stable lipid hydroperoxide (ROOH) thereby protecting the cell membrane by maintaining its integrity and stability (Fig. 3). During this process there is production of α- tocopheroxyl radical (α TO-) which is stable as compared to the other free radicals. This alpha tocopheroxyl radical is restored back to vitamin E in the cell by vitamin C, glutathione, ubiquinone and other pathways (Packer, et al., 1997).

Non antioxidant functions of vitamin E

Alpha tocopherol inhibits protein kinase C (PKC) activity, a signaling molecule that can regulate cell proliferation. This specific inhibitory effect on cell proliferation contributes to the anti-atherosclerosis and anti-tumor effects of vitamin E (Azzi, et al., 2002). Vitamin E also inhibits platelet aggregation by Protein kinase C dependent mechanism and prevents thrombus formation in the blood vessels (Azzi, et al., 2002). Vitamin E inhibits prostaglandin E2 production by macrophages, which has a suppressive effect on T cells (Meydani, et al., 1997, Meydani, et al., 1986).

Supplementation of vitamin E to old mice enhanced the expression of cell cycle related proteins that aid in entering into mitotic phase and help in DNA replication (Pelizon, 2003, Quadri, et al., 1998). In cancer cells, vitamin E was hypothesized to prevent the proliferation of cells by down regulating cell cycle regulatory proteins (Ni, et al., 2003, Turley, et al., 1997)

Vitamin E deficiency

A vitamin E concentration < 2ug/ml in serum/plasma is considered deficient (NRC, 2007). Vitamin E deficiency, alone or in conjunction with selenium deficiency, has been linked to several clinical syndromes that are variably manifested in a wide variety of species. The most common and well described clinical manifestation due to the deficiency of vitamin E and selenium is white muscle disease (WMD), also known as nutritional muscular dystrophy in lambs, calves, and foals (Abutarbush and Radostits, 2003). In lambs, the clinical signs due to WMD include weakness, stiffness, inability to stand and curved back (Deger, et al., 2008). Postmortem examination reveals necrosis, mineralization, and fibrosis of the skeletal and cardiac muscle (Beytut, et al., 2002, Bostedt and Schramel, 1990). Vitamin E deficiency is thought to be the cause of equine motor neuron disease and equine degenerative myeloencephalopathy in equines, exudative diathesis and encephalomalacia in poultry, mulberry heart disease and hepatitis dietetica in pigs, and hepatic necrosis in rats.

Previous studies have shown that a deficiency of vitamin E has negative impact on both the humoral and cell mediated immunity. A study on vitamin E deficient animals showed decreased natural killer cell (NK) activity and decreased

phagocytosis by polymorphonuclear cells (Moriguchi and Muraga, 2000). Pigs and dogs fed a diet deficient in selenium and vitamin E decreased lymphocyte proliferation to mitogens (Lessard, et al., 1993). A study in selenium and vitamin E deficient mice reported decreased resistance to gastrointestinal nematode infection (Smith, et al., 2005).

A deficiency in antioxidant nutrients like vitamin E and selenium can increase viral pathogenicity by decreasing immune response of the host which in turn can cause mutations in a benign virus transforming it into a virulent strain (Beck, et al., 1994, Beck, et al., 2005). This new aspect of nutrition altering viral pathogenicity was a breakthrough in nutritional immunology.

Toxicity of vitamin E

Evidence of vitamin E toxicity is rare. According to NRC, 2006 up to 70 IU/Kg body weight (BW)/day is considered safe. However, vitamin E during the process of scavenging free radicals forms tocopheroxyl radical which can act as prooxidant when given in large quantities and which, if not restored back by vitamin C or glutathione, can produce harmful effects (Bell and Grochoski, 2008). Recently, some researchers have reported increased mortality and cardiovascular diseases with higher levels of vitamin E supplementation in humans (Clarke, et al., 2008).

VITAMIN E AND IMMUNITY

The effect of vitamin E on disease resistance has been studied in a wide variety of animal species. Vitamin E has been shown to modulate both the innate and adaptive components of the immune system (Han, et al., 2006, Hogan, et al., 1992, Singh, et al., 2006, Tanaka, et al., 1979, Tengerdy, et al., 1973). Studies by Meydani

and Moruguchi demonstrated that vitamin E is required to maintain immune function in the elderly (Meydani, et al., 1990) and improve the decline in cellular immunity that occcurs with aging (Meydani, et al., 1986, Sakai and Moriguchi, 1997).

Vitamin E and innate immunity

Vitamin E supplementation has been shown to improve neutrophil function in cows during calving period (Hogan, et al., 1990, Hogan, et al., 1992). Vitamin E in combination with selenium increased neutrophil function in dairy cows (Gyang, et al., 1984). A study conducted on geriatric horses supplemented with parenteral vitamin E showed an increase in the level of bacterial killing in supplemented horses but lack of significant effect on serum lysozyme activity (Petersson, Unpublished data). Sows fed 72 IU of α- tocopherol/kg of feed, during pregnancy and lactation improved their piglets serum lysozyme concentrations at 1 week of age, but had a negative impact when they were fed with 202 IU/kg of feed (Babinszky, et al., 1991).

Selenium and vitamin E supplementation in the cow during late stages of pregnancy (Lacetera, et al., 1996) and during the periparturient period (Weiss, et al., 1990) increased GSH-Px activity in cows and their calves. Supplementation with 3000 IU/day of vitamin E during transition period in cows prevented the decrease in superoxide anion production in neutrophils and Interleukin-1 production when compared to control cows (Politis, et al., 1995). Rats fed 100-2500 mg/Kg diet of vitamin E showed increased phagocytic activity in alveolar macrophages, which was dose dependent (Moriguchi, et al., 1990). However, vitamin E did not influence the measures of mammary macrophage function in dairy cows (Politis, et al., 1995, Politis, et al., 1996). Incidence of clinical mastitis (Smith, et al., 1984, Weiss, et al.,

1997), retained placenta (Erskine, et al., 1997, Miller, et al., 1993) and metritis (Erskine, et al., 1997) was reduced in cows supplemented with oral or parenteral vitamin E. More recently, a study conducted on murine peritoneal macrophages showed that supplementation with vitamin E can safeguard the morphology of immune cells like macrophages from the effect of peroxidative agents (Kaur, et al., 2009). Studies supplementing vitamin E and selenium during dry period in ewes (Morgante, et al., 1999), reported an increase in blood glutathione peroxidase activity, but no significant effect on plasma lysozyme activity.

Vitamin E and Humoral immunity

In 1973, Tengerdy et al. supplemented mice with vitamin E (60-180 mg/Kg of feed) and reported a 30-40% enhancement in the humoral immune response against sheep red blood cells (SRBC) and tetanus toxoid antigens (Tengerdy, et al., 1973). In this study, they demonstrated that the increase in humoral immunity was the primary immune response, which includes early stage of immunocyte development and proliferation. Dietary supplementation of vitamin E (300 mg/kg diet) to chickens significantly increased survival rate upon challenge with *E. coli* and the protection was attributed to increased antibody production and increased phagocytosis (Tengerdy and Brown, 1977).

Studies during gestation period Supplementation with parenteral selenium (5mg/100 kg BW) and dl α-tocopherol acetate (25 IU/Kg BW) during the late stages of pregnancy, did not influence plasma IgG concentrations of cow or their calves (Lacetera, et al., 1996). In agreement with the previous study, pigs supplemented with vitamin E at 22- 88 IU/ kg feed during gestation and 55- 220 IU/kg feed during

lactation had no effect of vitamin E on total IgG, IgM, IgA in plasma, colostrum and milk of gilts and their piglets (Nemec, et al., 1994). Gentry et al. reported that lambs from ewes injected with 1500 IU vitamin E, 21 d before parturition, had greater concentrations of serum IgG than lambs born to control ewes (Gentry, 1992a). Conversely, supplementation of pregnant ewes with 400 IU of vitamin E/ewe/day, 32 days to 0 day prior to lambing, did not have any effect on ewe and lamb serum IgG (Daniels, et al., 2000).

Studies in young animals Calves supplemented with oral vitamin E and C alone or in combination did not produce any significant increase in IgG and IgM response (Hidiroglou, et al., 1995). The IgG results were in agreement with Reddy et al. in calves receiving 1400 -2800 IU of oral dl-α-tocopherol acetate and 1400 IU dl-α-tocopherol parenterally but IgM was significantly higher in the orally supplemented calves (Reddy, et al., 1986). In a recent study, neonatal calves supplemented with 2000 IU of parenteral vitamin E weekly, starting at birth until the age of two weeks, showed an increase in the total IgG concentration and IgM concentration on day 22 (Pekmezci and Cakiroglu, 2009).

Vitamin E supplementation and vaccination response

Vaccination is an important tool in controlling and eradicating a disease. Immunological responses to specific pathogens can be increased by vaccination. Hence, the greater the production of antibodies against an antigen, the greater the protection offered against the disease.

Research in pigs, chickens, and other animals

Dietary vitamin E supplementation at 100 IU/ Kg of feed to 6-8 wk old pigs yielded enhanced primary and secondary immune response against *E.coli* bacterin when compared to the pigs receiving the NRC recommended amount of 20 IU/Kg of feed and control pigs (Ellis and Vorhies, 1976). This increase in humoral response in vitamin E supplemented pigs was two to threefold greater than control pigs. Horses supplemented with vitamin E alone or a combination of vitamin E and selenium produced higher antibodies against tetanus toxoid and equine influenza virus (Baalsrud and Overnes, 1986). The findings of these studies are similar to a more recent study on geriatric horses supplemented with vitamin E, which showed increased antibody production against tetanus toxoid vaccination (Petersson, Unpublished data). Serum antibodies in chickens vaccinated against Newcastle Disease virus were associated with higher hemagglutination inhibition titer and total immunoglobulins in vitamin E and selenium supplemented chickens compared to an unsupplemented group (Singh, et al., 2006). Dogs supplemented with parenteral vitamin E and selenium showed increased antibody titer against *Taenia hydatigena* and IgG concentration compared to controls (Kandil and Abou-Zeina, 2005)

Research in Cattle

Calves supplemented with 4180 IU/animal of oral vitamin E at weekly intervals showed increased IgM levels (Reddy, et al., 1986) but no change in total IgG concentrations (Reddy, et al., 1986). Further work by the same group reported higher antibody titers to Bovine herpes virus-1 vaccine in the calves supplemented with vitamin E at 125 IU/day/calf compared to the calves receiving 250 IU, 500 IU and the

control calves (Reddy, et al., 1987). Parenteral supplementation of vitamin E and selenium to the steers enhanced their humoral immune response against *Pasteurella hemolytica* vaccination (Droke and Loerch, 1989). A combination of selenium (0.1 mg Se/kg bodyweight) and vitamin E (α-tocopherol acetate, 8 U/kg BW), to the cows during late pregnancy improved the production of specific antibodies against *E.coli* bacterin (Panousis, et al., 2001). A mixture of vitamin E and Freunds adjuvant resulted in higher antibody response in serum and milk against *E.coli* bacterin compared to Freund's alone as the adjuvant and control cows (Hogan, et al., 1993b).

Research in Sheep

Many of the studies evaluating the effect of vitamin E on immune response have investigated the influence on antibody production. Tengerdy et al. reported increased antibody response against *Clostridium perfringens* in sheep supplemented with 300 mg/Kg feed of dl α-tocopherol acetate (Tengerdy, et al., 1983). In the same study, they reported that adjuvant vaccine containing a fraction of vitamin E was more effective in producing antibody response than the oral supplementation. (Tengerdy, et al., 1983). In agreement with this study, vitamin E when used as an adjuvant with *Brucella ovis* vaccine provided greater protection to rams against epididymitis in comparision with Freunds incomplete adjuvant or commercial bacterin vaccinated rams (Afzal, et al., 1984, Tengerdy, et al., 1991). Vitamin E supplementation to sheep at 1000 IU/animal orally in the form of capsules did not prevent infection with *Chlamydia* or development of typical lung lesions, but it appeared to speed the recovery from infection (Stephens, et al., 1979).

Increased antibody response against parainfluenza-3 virus was seen in sheep supplemented with a combination of selenium (0.2mg/Kg feed) and vitamin E (30 IU/Kg feed) (Reffett, et al., 1988). In contrast to this study, sheep supplemented with selenium (0.1 mg/kg BW) had a significant increase in *Chlamydia* antibody response but no effect was seen when selenium was given in combination with vitamin E (Giadinis, et al., 2000). Also, supplemental vitamin E (d-α-tocopherol acetate, 400 IU/ewe/day) to the ewe during gestation period had no effect on the response to vaccination against parainfluenza-3 virus (Daniels, et al., 2000).

Vitamin E and cell mediated immunity

Supplementation of vitamin E (800 mg dl-alpha-tocopheryl acetate for 30 d) to healthy elderly subjects produced an increase in interleukin-2 production and mitogenic response to concanavalin A (Meydani, et al., 1990). Another study in elderly patients supplemented with vitamin E, significantly improved age-related early T cell signaling events in naive CD4(+) T cells (Marko, et al., 2007). Increased T helper cell activity was found in mice supplemented with vitamin E (Tanaka, et al., 1979). Vitamin E has been shown to inhibit prostaglandin E_2 production from macrophages enhancing cellular immunity (Moriguchi, et al., 1990, Romach, et al., 1993). Calves supplemented with vitamin E showed increased lymphocyte proliferation when stimulated with mitogens (Reddy, et al., 1987). In agreement with this study, vitamin E and selenium supplementation to pigs (Larsen and Tollersrud, 1981) and sheep (Larsen, et al., 1988b) also enhanced lymphocyte proliferation upon mitogenic stimulation. However, Politis et al. did not find any effect of vitamin E on lymphocyte proliferation in cows (Politis, et al., 1995).

CONCLUSION

Results of studies examining the effects of vitamin E supplementation in ruminants are variable and limited, but have shown enough benefits for additional investigation. Research undertaken in this dissertation is intended to enhance the evolving database of knowledge on vitamin E supplementation in late gestation sheep and its effects on innate and humoral functions of ewes, their newborns and vaccination response.

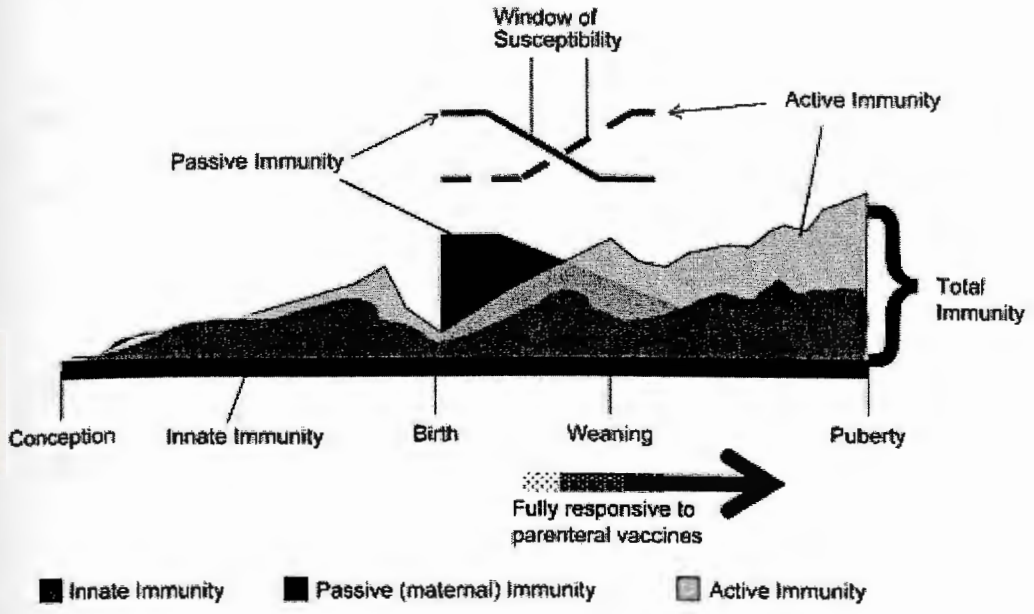
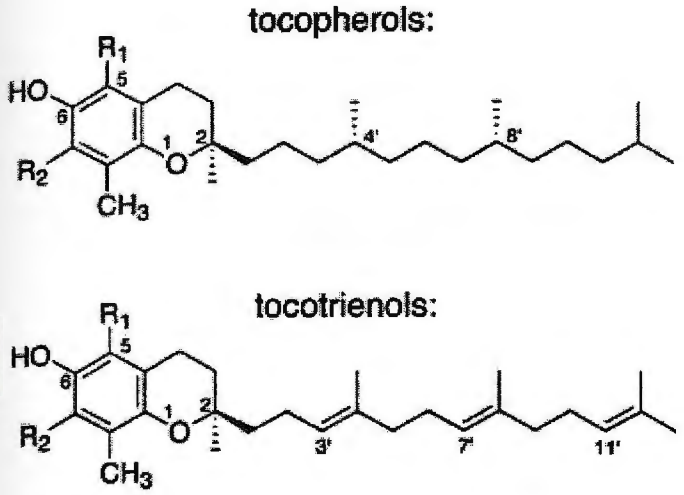


Fig. 1.1. Development of the immune response in the calf. (*Data from* Morein B, Abusugra I, Blomqvist G. Immunity in neonates. Vet Immunol Immunopath 2002;87:207–13; and Butler JE, Sinkora M, Wertz N, et al. Development of the neonatal B and T cell repertoire in swine: implications for comparative and veterinary immunology. Vet Res 2006 37:419.)



tocopherol/ tocotrienol	R_1	R_2	relative antioxidant activity (%) in vitro
α-	CH_3	CH_3	100
β-	CH_3	H	71
γ-	H	CH_3	68
δ-	H	H	28

Fig. 1.2. Structure of Tocopherols, Tocotrienols, relative antioxidant activity of their isoforms. Taken from Schneider, 2005.

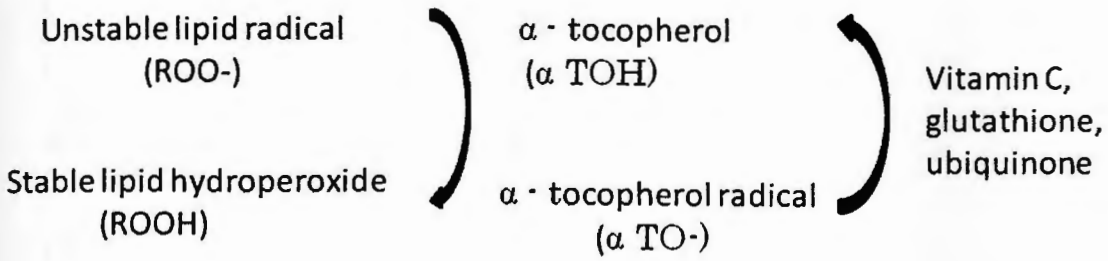


Fig. 1.3. Antioxidant action of vitamin E. Adapted from Packer et al, 1997.

Table 1.1. Summary of literature on vitamin E in cattle

Study subjects	VE dose	Route	Duration	Effect	Authors
Calves	2000IU/day/weekly	Parenteral	At birth, 7 and 14 d of life	IgM at day 22 of age: ↑ IgG: ↑	Pekmezci et al (2009)
Calves	VE-900, 1800, 2700 IU	Parenteral	At birth upto 12 wks of age (3 wk interval)	IgG – Titer to KLH – At 2700 IU IgM:↑	Hidiriglou et al (1992)
Pregnant cows	Se(0.05 mg/kgBW) and VE (0.25IU/kgBW)	Parenteral	3 & 1.5 wks before calving	GSH-Px of dam & calves: ↑ IgG of dam & calves –	Lacetera et al (1996)
Pregnant Cows	3000 IU VE	Parenteral	at 10 d and 5 d prior to claving	Neutrophils killing ability: ↑	Hogan et al, (1992)
Calves	125 IU/day/calf	oral	Birth to 24 wks of age	IgG against BHV1: ↑	Reddy et al (1987)

Table 1.2. Summary of literature on vitamin E in sheep

Study subjects	VE dosage	Route	Duration	Effect	Authors
Pregnant sheep	400 IU/ewe/day, daily	oral	32 d before lambing	Ewe, lamb, colostral IgG – Anti PI3 titers –	Daniels et al (2000)
Pregnant sheep	1500 IU/ewe	Parenteral	21 d before lambing	Lamb Serum IgG: ↑ Colostral IgG: –	Gentry et al (1991)
Lambs	30 IU/Kg feed (acc NRC 1985)	oral	70 d period from the age of 2 mts During vaccn period	Serum IgG – Anti PI3 titers:↑sec immune response GSH-Px activity: ↑	Reffet et al (1988)
Pregnant sheep	75 IU/day/sheep	oral	4.5 mts prior to parturition	Serum & colostral IgG – Anti tetanus toxoid: ↑	Larsen et al (1988)
Sheep	1500 IU/ewe	oral	6 weeks Weekly interval	Chlamydia psitaci titres –	Giadnis et al (2000)

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CHAPTER 2:

Influence of Vitamin E on Innate and Humoral components of the Immune system of Pregnant Ewes and their Lambs

ABSTRACT

The effect of parenteral administration of vitamin E during the pregnancy and lactation period of ewes and their lambs on innate and humoral components of the immune system was investigated in a 3 part study. 1.) Pregnant/Lactating ewes: Fifteen pregnant Dorset sheep were supplemented with vitamin E (30 IU d-α-tocopherol /Kg body weight (BW), E, n=10) or placebo (emulsified base, P, n=5) from the last trimester of gestation through weaning. Blood and colostrum were analyzed every 2 wk for α-tocopherol, selenium, glutathione peroxidase activity, lysozyme activity, total IgG and IgG antibodies against tetanus toxoid. 2.) Lambs (0-8wks): Lambs born to E and P supplemented ewes received either vitamin E (30 IU d-α-tocopherol /Kg BW) or placebo (emulsified base) every 2 weeks from birth through 8 wk of age. This resulted in four treatment groups: E/E (n=10), E/P (n=11), P/E (n=4), P/P (n=5). The lambs were sampled every other week and their blood was analyzed as for the ewes. 3.) Lambs (Vaccination period): When the youngest lamb reached 8 wks of age all lambs were vaccinated as a group against *Clostridium perfringens* and *Clostridium tetani*. Booster vaccinations were given 4 wk later. Blood was sampled prior to primary vaccination, 1 wk post vaccination and then every 2 wk until 7 wk post vaccination. Results: Pregnant/Lactating ewes: Serum vitamin E concentrations increased over time in supplemented ewes as compared to placebo ewes. Colostrum

vitamin E levels were also increased in supplemented versus placebo ewes. Vitamin E supplementation did not influence plasma selenium, glutathione peroxidase activity and serum lysozyme, IgG and IgG against tetanus toxoid in the serum or colostrum of ewes. There were fluctuations within treatment in the production of IgG in response to parturition. Lambs (0-8wks): Lambs directly supplemented with vitamin E in addition to maternal supplementation (E/E) had higher levels of serum α-tocopherol than the placebo group (P/P). There were no differences in plasma selenium, glutathione peroxidase activity, serum lysozyme, IgG and IgG against tetanus toxoid concentrations between groups. There was a loss of maternal antibodies over time in all lambs. Serum lysozyme activity was higher at birth, prior to ingestion of colostrum, in lambs that received maternal vitamin E supplementation compared to the lambs that received only direct vitamin E supplementation. Lambs (Vaccination period): Serum α-tocopherol levels increased in lambs that received direct vitamin E supplementation (E/E and P/E) irrespective of maternal supplementation (P/P and E/P). Plasma selenium, glutathione peroxidase activity, serum lysozyme and IgG did not differ between treatment groups. There was an increase in the production of IgG against tetanus toxoid in response to booster vaccination in PE lambs. Conclusions: Supplemental vitamin E to the ewes and lambs, at 30 IU/Kg BW, had a modest effect on parameters used in this study to assess innate and humoral immunity.

(**Key words:** vitamin E, ewe, IgG, tetanus toxoid)

INTRODUCTION

Vitamin E has been shown to modulate immune function in several species of animals (Meydani, et al., 1986, Reddy, et al., 1987, Tengerdy, et al., 1983). The maternal immune system during the transition period is under increased physiological stress. The stress during pregnancy and increased metabolic demands during parturition and lactation result in increased production of reactive oxygen species leading to oxidative stress and a subsequent reduction in neutrophil function, antibody responses and cytokine production by immune cells (Spears and Weiss, 2008). Efforts to enhance immune function of pregnant animals through vitamin E supplementation have been met with varied results (Daniels, et al., 2000, Gentry, 1991, Giadinis, et al., 2000, Larsen, et al., 1988).

Maternal immune function will impact their offspring through maternal transfer of immune cells in the colostrum. The immune response of lambs to maternal vitamin E supplementation has been varied (Daniels, et al., 2000, Gentry, 1992) with either no effect (Capper, et al., 2005, Daniels, et al., 2000) or an increased effect (Gentry, 1992) on serum immunoglobulin G (IgG) reported.

It has been generally assumed that there is no significant transfer of vitamin E across the placenta in ruminants (Njeru, et al., 1994). Recently however, supplementation of dietary vitamin E to ewes from 6 wks prepartum to 6 wks postpartum enhanced serum α-tocopherol levels in muscle tissue of lambs at birth even though plasma tocopherol concentration did not differ between groups (Capper,

et al., 2005). The potential of vitamin E supplementation of the ewe and her offspring to enhance immune function is an area that warrants further study.

Therefore, the present experiment was designed to determine the effect of: 1.) Vitamin E supplementation on immune function in pregnant and lactating ewes; 2.) Direct vitamin E supplementation to the lamb superimposed on maternal supplementation on innate and adaptive immune function in lambs from birth through 7 months of age.

MATERIALS AND METHODS

Animals

Pregnant/lactating Dorset ewes (n=18) ranging from 1-9 yrs of age and their lambs (n=30) were included in this study. All the ewes and lambs were maintained, treatments were administered, and samples were collected in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Rhode Island. Ewes were fed with hay and grain whereas lambs had a free choice of feed. Feed analysis for various minerals, vitamin E and selenium are shown in Table 2.1. Body condition and weight were monitored for the ewes and lambs throughout the study.

Study Design

Pregnant/Lactating Ewes. Pregnant Dorset sheep were randomly assigned to receive either vitamin E (30 IU d-α-tocopherol /kg body weight (BW), E, n=10) or placebo (P, n=8). The mean age of the ewes was 3.9 (±0.75) and 5.5 (±0.89) for vitamin E and placebo groups respectively. Intramuscular injections of d-α-tocopherol (Neogen, Lexington, KY) or equivalent quantity of placebo (emulsified base) were given every two weeks from the last trimester of gestation until weaning of

lambs at 3-4 months of age. Ewes were administered a yearly booster vaccination for *Clostridium tetanus* and *Clostridium perfringens* (C+D) (Colorado Serum Company, Denver, CO) approximately 6 weeks prior to parturition.

Lambs (0-8wks). At birth, lambs born to vitamin E or placebo supplemented ewes, were assigned to receive either vitamin E (30 IU d-α-tocopherol/kg BW) or placebo (emulsified base) intramuscular injections from birth through 8 wk of age. This resulted in four treatment groups: 1.) Maternal E, Lamb E (E/E, n=10) 2.) Maternal E, Lamb P (E/P, n=11) 3.) Maternal P, Lamb E (P/E, n=4) 4.) Maternal P, Lamb P (P/P, n=5). Following standard management practices all lambs received a single sodium selenite injection (0.1mg/kg BW) at 1 day of age. Body weight was monitored every two weeks throughout the study.

Lambs (Vaccination). When the youngest lamb reached 8 weeks of age, all lambs were vaccinated as a group against *Clostridium tetanus* and *Clostridium perfringens* C+D (Colorado Serum Company, Denver, CO). Secondary vaccinations were administered 4 weeks later. The lambs were transitioned to receive their biweekly injections of vitamin E.(30 IU/Kg BW) or placebo (equivalent mls/Kg BW) as a group until the end of the study. Blood from lambs after vaccination was collected on the day of initial vaccination, 1 week later and every two weeks until the end of study. Lambs ranged from 8-14 wks of age at this start of this study period.

Sample collection

Blood samples were collected via jugular puncture from ewes every two weeks throughout the study period and from lambs, at birth (prior to colostrum consumption), weekly until one month of age and then every two weeks until 8 weeks of age. All

blood samples were collected prior to biweekly injections of d-α-tocopherol or placebo. All samples were analyzed for α-tocopherol, selenium and glutathione peroxidase (GSH-Px) as a measure of antioxidant status, lysozyme as a measure of innate immunity, and IgG, and anti-tetanus toxoid IgG (IgGt) as a measure of humoral immune status. Lamb weights were measured to monitor growth performance.

Blood was collected into a serum separator and EDTA vacutainers (Becton Dickinson, Franklin Lakes, NJ) for serum and plasma respectively. Tubes were centrifuged (1750 × g) at 25° C for plasma and 4° C for serum. Colostrum from the ewes was collected immediately after parturition. All the samples were stored at -80° C prior to analysis colostrum was centrifuged at 24,400 g for 20 minutes at 4° C, the fat layer removed, and the whey fraction was analyzed for lysozyme, IgG and IgG against tetanus toxoid.

Alpha Tocopherol in Serum and Colostrum

Samples were analyzed for α-tocopherol content using established procedures at the Diagnostic Center for Population and Animal Health (DCPAH, Lansing, MI). Briefly, reverse phase HPLC (Hitachi, Pleasanton, CA) with a 3.5 um C18 column (Waters Corp, Milford, MA) and acetonitrile: methylene chloride: methanol mobile phase (70:20:10) was used. Alpha tocopherol was detected at a wavelength of 292 nm and results are expressed as μg/ml.

Selenium and GSH-Px in Plasma and Colostrum

Samples were analyzed at the USDA Grand Forks Human Nutrition Research Center (Grand Forks, North Dakota) for selenium content and GSH-Px activity. Selenium concentrations were determined with electrotherma atomic absorption

spectrophotometry using a reduced Pd matrix modifier according to published procedure (Clark, et al., 1996) and results are expressed as ng/ml. GSH-Px was assayed by the glutathione reductase-coupled assay (Paglia and Valentine, 1967) as modified by Lawrence and Burke (Lawrence and Burk, 1976) using 0.25 mM H_2O_2 as substrate. Results are expressed as nanomoles of NADPH/min/mg of protein.

Lysozyme Activity

Lysozyme activity was determined by the radial diffusion of samples through a suspension of live culture of *Micrococcus luteus* (ATCC 27141, Manassas, VA) in M/15 phsophate buffered agarose, pH 6.3 following published procedure (Sotirov, 2005). The diameters of the lytic zones were compared with the lytic zones of the standard, hen egg white lysozyme (Sigma Aldrich, St.Louis, MO). Results are expressed as µg of hen egg white lysozyme equivalent per ml.

IgG

A commercial ELISA kit (Bethyl Labs, Montgomerry, Texas) was used to detect and quantify sheep IgG antibodies in the serum and colostrum. Briefly, rabbit antisheep IgG antibody is coated onto plates and incubated followed by blocking with 0.05M tris buffered saline (Sigma-aldrich, St.Louis, MO) and addition of standard or serum sample. Rabbit antisheep IgG labeled with horse radish peroxidase enzyme was used as the detection antibody. Reaction terminated using stop solution (2 M H_2So_4) and the absorbance is read at 450 nm. Results are expressed as mg/ml of serum.

IgG against tetanus toxoid

Antibodies to tetanus toxoid were determined according to (Rosskopf, et al., 2005) with some modifications. Briefly, 0.2 lf/ml (limit of flocculation units) of

commercially available tetanus toxoid was coated onto 96 well plates and incubated overnight at 4°C. Wells were blocked with phosphate buffered saline (PBS), followed by addition of standards and samples diluted in PBS (0.01 M with 0.05 % tween 20). Rabbit anti-sheep IgG horse radish peroxidase conjugated antibody (Bethyl Labs, Montgomerry, Texas) was used as developing antibody, followed by the addition of tetramethylbenzidine (TMB) susbtrate to the plates and incubated in dark for 15 minutes before the reaction was terminated by 1.25 M H_2SO_4. The results are expressed as absorbance units at 450nm (OD^{450}).

Statistical analysis

The mixed procedure in SAS (SAS Inst. Inc., Cary, NC) was used to analyze serum α-tocopherol, lysozyme activity, antibody concentrations, selenium, and GSH-Px. The random effect of ewe or lamb was accounted for with a repeated measures approach. All outcome variables were assessed for normality. A logarithmic transformation was applied to non-normally distributed data. The appropriate error structure was assessed and applied for each outcome variable. Independent variables offered into each model included age, sex, treatment, and time. The interaction of treatment and time was evaluated for each model. Variables were removed by backwards elimination based on order of least significance. Tukey's adjusted *P*-values were calculated for each variable to adjust for multiple 2-way comparisons within each model. Once the final model was chosen, residuals were checked for homoscedasticity, outliers, leverage cases, and normal distribution. Student's t-test was used to statistically analyze colostral content of α-tocopherol, selenium, GSH-Px

activity, lysozyme activity, IgG and IgGt. Significance was defined for all tests as $P \leq 0.05$.

RESULTS

Three ewes from the placebo group, though testing positive on ultrasound, did not lamb and therefore were removed from the study. This resulted in 5 animals in the placebo group (5.8±1.11 yrs) and 10 animals in the vitamin E group (3.9±0.75 yrs). Feed analysis of the mixed grass hay and grain fed to sheep and lambs is shown in Table 2.1.

Pregnant/Lactating Ewes.

Serum α-tocopherol increased over time in vitamin E supplemented ewes ($P \leq 0.001$, Fig. 2.1) as compared to placebo supplemented ewes. There was also a treatment * week interaction for serum IgG ($P \leq 0.05$, Fig. 2.2). Placebo supplemented ewes had a greater concentration of plasma selenium than vitamin E supplemented ewes ($P \leq 0.05$, Table 2.2). There was no effect of treatment or treatment by week interactions for plasma GSH-Px activity, serum lysozyme activity and serum IgGT. The colostrum from vitamin E supplemented ewes contained greater ($P \leq 0.05$, Table 2.2) amounts of α-tocopherol than placebo supplemented ewes. There was no effect of vitamin E supplementation on colostral selenium, GSH-Px activity, lysozyme activity and IgG against tetanus toxoid concentrations. Treatment means (+/- SE) for all variables is shown in Table 2.2.

An increase in IgG concentrations were seen in vitamin E supplemented ewes after their yearly booster vaccination against *Clostridium tetanus* and *Clostridium perfringens* ($P \leq 0.05$, Fig. 2.2). There was no vaccination response in the placebo

supplemented ewes. There was a decrease in serum IgG in both vitamin E and placebo supplemented ewes during the transition period that was more pronounced in the placebo supplemented ewes ($P \leq 0.05$, Fig. 2.2).

Plasma selenium, plasma GSH-Px activity, serum lysozyme activity, and serum IgGT increased over the length of the study period ($P \leq 0.001$, Table 2.3).

When the effect of ewe age on the statistical model was determined plasma selenium concentrations were higher ($P \leq 0.05$) in younger ewes ($\leq$ 5yrs) as compared to older ewes ($\geq$ 7 yrs) and serum IgGT concentrations was greater in older than younger ewes. There was no effect of age on serum α-tocopherol, lysozyme activity, IgG and plasma GSH-Px acitivity. The effect of age for all the variables is shown in Table 2.4.

Lambs (0-8wks).

Serum α-tocopherol was greater in EE lambs than PP lambs ($P \leq 0.05$, Fig. 2.3) Lambs receiving maternal vitamin E supplementation but no direct supplementation (E/P) had elevated serum α-tocopherol over that of P/P that did not reach the level of significance (P = 0.0685). At birth, before suckling serum lysozyme activity was higher ($P \leq 0.001$, Fig. 2.4) in lambs that received maternal vitamin E supplementation compared to the lambs whose dams received placebo. There was no effect of treatment or treatment by week interaction for the plasma selenium and GSH-Px activity, and serum IgG and IgGT. Mean (+/- SE) of all variables by treatment is shown in Table 2.5.

There was an effect of week ($P \leq 0.001$, Table 2.6) for serum vitamin E, IgG, IgGT, plasma selenium and GSH-Px activity. Serum IgG and IgG against tetanus

toxoid decreased over time in all lambs regardless of supplementation. Glutathione peroxidase and plasma selenium concentration increased over the length of the study.

Lambs (Vaccination Period).

Serum α-tocopherol increased over time in lambs receiving direct vitamin E supplementation (EE and PE) as compared to lambs supplemented with placebo (EP and PP, $P \leq 0.001$, Fig. 2.5). Secondary vaccination against *Clostridia tetani* elicited a strong response in lambs receiving direct vitamin E supplementation but no maternal supplementation (PE, $P \leq 0.001$, Fig. 2.6). There was no significant increase in production of antibody production in response to the secondary vaccination against *Clostridia tetani* in all other treatment groups. There was no effect of treatment or treatment by week interaction for the variables, selenium, GSH-Px activity, lysozyme activity and IgG. Mean ± SEM for all the variables by treatment is shown in Table 2.7.

There was an effect of week of study ($P \leq 0.001$, Table 2.8) for plasma selenium, GSH-Px activity, serum IgG and IgGT. Glutathione peroxidase activity and plasma selenium decreased in all lambs during the vaccination period. There was an increase in total serum IgG levels following primary and secondary vaccination in all the lambs.

When the effect of lamb age, at the start of the vaccination period, on the statistical model was determined plasma selenium concentration were greater ($P \leq 0.05$) in older lambs (≥ 12 wks) compared to younger (≤ 12 wks) lambs. We did not observe an effect of age on serum α-tocopherol, lysozyme activity, IgG, IgGT and plasma GSH-Px acitivity. Mean ± SEM by age of lamb at the start of the vaccination

period is shown in Table 2.9. When the effect of lamb sex on the statistical model was determined ewe lambs had a higher concentration of serum α-tocopherol (1.29 ± 0.06 μg/ml, $P \leq 0.001$) than ram lambs (0.98 ± 0.05 μg/ml).There was no effect of sex for any other study variables.

DISCUSSION

Pregnant/Lactating Ewes

As expected, there was an increase in serum α-tocopherol levels in supplemented versus placebo ewes. Serum or plasma α-tocopherol levels are routinely used as indicators of vitamin E status. Serum α-tocopherol concentrations > 4 μg/ml are considered adequate, 2-4 μg/ml is marginal and animals with < 2 μg/ml are considered deficient according to cattle studies (Bass, et al., 2001). In our study, we observed marginally deficient tocopherol levels at baseline in all ewes and in placebo ewes throughout the study. Vitamin E supplemented ewes reached the 4 ug/ml threshold by the end of the study.

Placebo ewes had higher plasma selenium levels compared to vitamin E supplemented ewes. The ewes in both the treatment groups were having adequate levels of selenium in their plasma (>100ng/ml). Vitamin E did not influence plasma GSH-Px activity but the activity reached peak around parturition. This increase may be due to the increased oxidative stress around parturition. The plasma GSH-Px activity levels are in agreement with those of Reffet et al. (1988) in lambs who received selenium supplementation. Ewes supplemented with parenteral vitamin E (7.5 IU/Kg BW/2 wks) and selenium (0.1mg/Kg BW/2 wks) one month before lambing reported an increase in blood GSH-Px activity (Morgante, et al., 1999). The

vitamin E supplemented in this study was less than the dose of supplemental vitamin E administered in our study. The differences in the GSH-Px activity can be attributed to the selenium injections given before lambing. As selenium is an integral part of the enzyme GSH-Px, supplemental selenium might have a direct influence on the GSH-Px activity in the morgante et al. study. In the present study, selenium and GSH-Px activity were measured to assess antioxidant status of the animal as the soil in northeast regions are considered as deficient in selenium levels. However, the ewes in this study were not deficient in selenium levels.

To date, there has been very little research on the effects of vitamin E on innate immunity in ruminants. In the present study, vitamin E had no influence on serum lysozyme activity. A similar trend was observed in ewes supplemented with vitamin E (7.5 IU/Kg BW/2 wks) and selenium (0.1mg/Kg BW/ 2wks) one month before lambing (Morgante, et al., 1999) and supplementation with vitamin E (15 IU/kg BW) to horses (K.H. Petersson, Unpublished data). The above reported studies administered lower dose of supplemental vitamin E compared to our study. Lack of significant effect on serum lysozyme concentrations in the present study suggests that oxygen independent killing mechanisms are insensitive to vitamin E status (Turner and Finch, 1990).

Although there was no effect of vitamin E supplementation on serum IgG levels, the ewes in both the treatment groups behaved differently over time. Placebo ewes did not show an increase in IgG levels against *Clostridium tetanus* and *Clostridium perfringens* vaccination, whereas vitamin E supplemented ewes showed vaccination response. There was decrease in IgG levels around parturition for both the

groups, but the immunosuppression occurring during this period appeared to be diminished in the vitamin E supplemented ewes compared to placebo ewes. Lack of treatment effect can possibly be due to the small sample size of the placebo ewes which might have masked the effect of vitamin E during parturition. In agreement with our findings, Daniels et al. (2000) reported that oral vitamin E (400 IU/ewe/day) supplementation to the ewes during last trimester of gestation had no effect on serum IgG levels. This is in agreement with most of the studies conducted in cattle (Lacetera, et al., 1996) sheep (Larsen, et al., 1988, Reffett, et al., 1988) with varying doses of supplemental vitamin E.

As expected, ewes vaccinated against tetanus toxoid produced greater amounts of tetanus specific antibodies in their serum. However, supplemental vitamin E did not influence the production of IgGT antibodies. These results were not in agreement with Larsen et al. (1988), who reported an increase in antibody titer to tetanus toxoid in ewes with dietary vitamin E supplementation (75 IU of α-tocopherol acetate/day). Although, the dose of supplemental vitamin E used in our study was higher than that of Larsen et al. we did not see any enhancement in the antibody titer. Plausible explanation for this difference could be the ewes in Larsen et al. study started with much lower levels of serum tocopherol and were less than 2 μg/ml throughout the study. And the supplemental vitamin E given at this period might have increased the production of antibodies. Also, the ewes were younger in Larsen et al. study compared to our study where we had a range of ewes from 1-9 yrs of age.

The primary source of the nutrients for the ruminant newborns at birth is through colostrum. Several studies (Bass, et al., 2001, Pinelli-Saavedra, et al., 2008, Weiss, et

al., 1990) have shown that vitamin E supplementation to the dam during gestation enhances α-tocopherol concentration in their colostrum. These results were in agreement with the present study. As placental transfer of immunity is limited in ruminants, ruminant newborns are entirely dependent on their dam's colostrum for the immunoglobulins. Transfer of adequate amounts of IgG from dams to newborns minimizes the risk of infectious diseases until their own immune system comes into play. However, in the present study, supplementing vitamin E to the ewes during last trimester did not influence their colostral IgG and IgG against tetanus toxoid levels. No effect of supplemental vitamin E on colostral IgG in the present study agrees with the results reported in ewes supplemented with vitamin E (Daniels, et al., 2000) and cattle (Lacetera, et al., 1996) during late gestation. Also, supplemental vitamin E to ewes during the last trimester did not influence colostral lysozyme activity, selenium and GSH-Px activity.

Lambs (0-8wks)

Serum α-tocopherol levels were higher in lambs that were directly supplemented with vitamin E in addition to maternal supplementation (E/E) compared to placebo (P/P). The lambs receiving only maternal supplementation (E/P) tended to have higher means but did not reach significance level due to the small sample size of the placebo lambs. This increase at week 1 can be due to the ingestion of colostrum rich in vitamin E from supplemented animals. Interestingly, Capper et al. (2005) reported increased tissue vitamin E levels in lambs at birth born to supplemented ewes, even though their serum vitamin E levels were in low range. As we did not sacrifice any lambs at birth we do not know whether the tissue content of vitamin E

was greater in the maternally supplemented lambs. The serum α-tocopherol concentrations in this study were in the deficient range; however, it is feasible that the supplemental vitamin E was being stored in the tissues but not reflected in the serum values. Further research is warranted in this area.

The selenium concentrations were low at birth but gradually increased over time as the lambs were feeding on grain which has selenium. There was a treatment by week interaction for the serum lysozyme activity in lambs. The lambs that were born to supplemented ewes had higher serum lysozyme activity at birth compared to the lambs that received only direct vitamin E supplementation. And the lambs were having higher means for serum lysozyme activity at birth compared to the rest of the life. This suggests that there might be increased lysozyme production in the fetal stage which might be reflected in the serum levels at birth before taking colostrum. And the vitamin E supplementation to the ewe might have influenced the production of lysozyme within the fetus.

IgG and IgGT antibodies were not detectable at birth due to limited placental transfer, but reached peak concentrations after the ingestion of colostrum at week 1 of age. Thereafter, the maternal antibodies decreased overtime in spite of vitamin E supplementation. During neonatal life, greater amounts of serum IgG has been positively correlated with higher survival rates. Supplementing vitamin E to the newborns may help in reducing morbidity due to pathogens during the critical period from the loss of maternal antibodies to the production of antibodies by the newborns themselves. There is controversy in the literature on the effects of supplemental vitamin E on humoral immune response. Study by Gentry et al. (1992) reported that

lambs from ewes injected with vitamin E, 21 d before parturition contained greater concentrations of serum IgG than lambs born to control ewes. Gentry et al. explained that though vitamin E did not influence colostral IgG levels, it might have influenced the lambs ability to absorb immunoglobulins in the gut. However, we did not see such an effect and our results were in agreement with the studies supplementing vitamin E to ewes (Daniels, et al., 2000, Reffett, et al., 1988), cows (Lacetera, et al., 1996) and pigs (Nemec, et al., 1994) during gestation which did not influence their newborns serum IgG levels. All of these studies vary in dose of supplemental vitamin E, interval at which they are administered, breed and species of animal.

Lambs (Vaccination Period)

Serum α-tocopherol levels were higher in lambs that were directly supplemented with vitamin E. The α-tocopherol concentrations in the E/E group differed from E/P group on the initial day of vaccination but the rest of the groups did not differ from each other on the initial day. The lambs that were supplemented directly with vitamin E had significantly higher levels of tocopherol compared to the lambs that received only maternal supplementation and the placebo lambs. This suggests that, the supplementation to the ewes during last trimester did not have a long term influence on their lamb's serum α-tocopherol levels. Also, the lambs that did not receive any supplementation (PP, EP) were having < 1μg/ml of serum tocopherol, which is considered deficient. The lambs that received direct vitamin E supplementation had serum tocopherol levels ranging between 1-2.5 μg/ml which is considered as marginal to adequate. Hence, vitamin E supplementation helped the lambs to maintain adequate vitamin E status.

Vitamin E did not influence plasma selenium and GSH-Px activity. Although, there were no significant interactions for serum lysozyme activity the means of the lambs that received only direct vitamin E supplementation tended to be higher than the other groups. The lambs showed enhanced total IgG levels against both the primary and secondary vaccination against *Clostridium tetanus* and *Clostridium perfringens* but were not influenced by treatment. Reffet et al., (Reffett, et al., 1988) reported that dietary supplementation of vitamin E to the lambs that were challenged with Parainfluenza-3 virus did not show any significant effect on total serum IgG levels.

To date, there are very limited studies in supplementing vitamin E to the small ruminants and its influence on antibody production to tetanus toxoid vaccination. There was no treatment effect but the lambs that were directly supplemented with vitamin E showed greater secondary immune response. Lack of significance might be due to insufficient power from small sample size in P/E lambs.

In summary, supplementing vitamin E parenterally during late gestation, direct supplementation to lambs did not appear to enhance the immune functions of sheep or the lambs in this study. Based upon these findings, parenteral vitamin E supplementation at the rate of 30 IU/kg BW/2 wks which is equal to 2 IU/kg BW/day to the ewes and lambs does not appear to be justified in improving their disease resistance. Since the study has started, the recommendations by NRC for vitamin E have been revised in 2007 (NRC, 2007) and the supplemental dosage is increased to 10 IU/Kg BW/day to protect the small ruminants from infectious diseases and to extend the storage life of meat. Hence, it can also be possible that the vitamin E supplementation used in this study might not be sufficient to elicit a significant

immune response. Role of supplemental vitamin E in influencing immunocompetence might be more pronounced when the animals are undergoing environmental and pathogenic stress. Therefore, future studies taking small ruminants with sufficient sample size and challenged with parasitic or bacterial pathogens might give a better understanding of the effects of vitamin E on innate and humoral immune parameters.

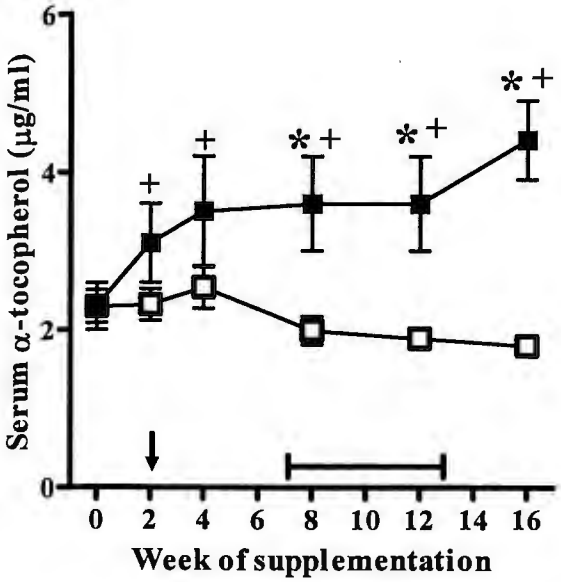

Fig. 2.1. Mean (± SEM) serum α-tocopherol concentrations of pregnant/ lactating ewes supplemented with d-α-tocopherol (■, 30 IU d-α-tocopherol /kg BW/2wks, n=10) or placebo (□, emulsified base, n=5). ↓ represents vaccination against *Clostridium tetanus* and *Clostridium perfringens*. ⊢—⊣ represents lambing. * represents $P<0.001$ vs placebo within week. + represents $P<0.001$ vs baseline within treatment.

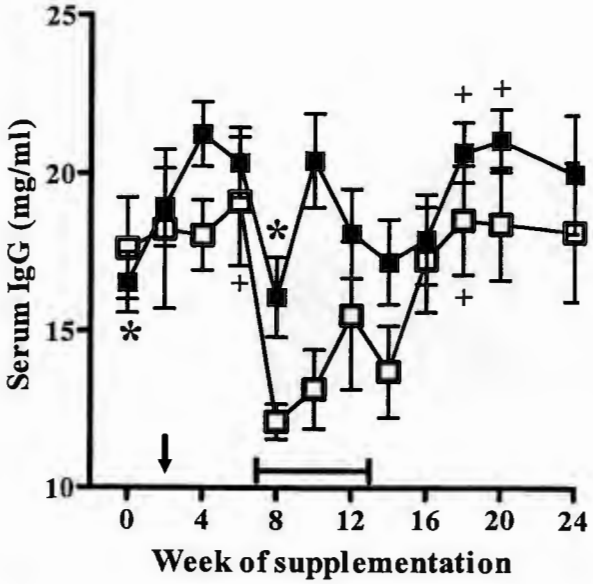

Fig. 2.2. Mean (± SEM) serum IgG concentrations of pregnant/ lactating ewes supplemented with d-α-tocopherol (■, 30 IU d-α-tocopherol /kg BW/2wks, n=10) or placebo (□, emulsified base, n=5). ↓ represents vaccination against *Clostridium tetanus* and *Clostridium perfringens*. ⊢—⊣ represents lambing. * represents $P<0.05$ vs wk 4 within treatment. + represents $P<0.05$ vs wk 8 within treatment.

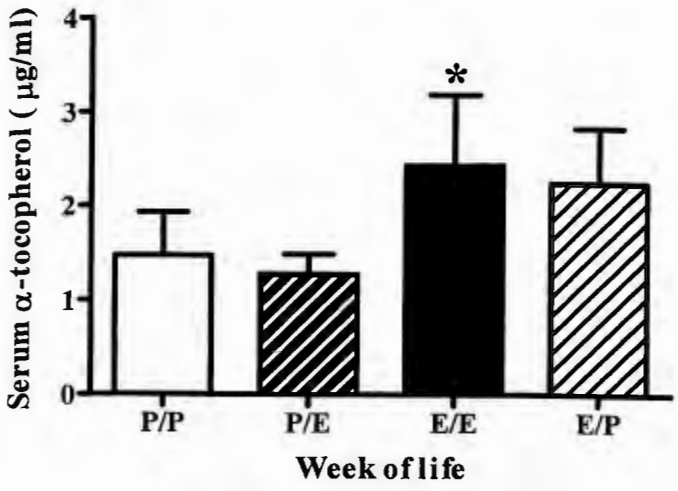

Fig. 2.3. Mean (± SEM) serum tocopherol concentrations in lambs from 0-8 wks of life averaged over time. Vitamin E supplementation was given at 30 IU/kg BW/2 wks or Placebo, emulsified base. Maternal vitamin E (E) Lamb E (E/E, n=10), Maternal E, Lamb placebo (P) (E/P, n=11) Maternal P, Lamb E (P/E, n=4), Maternal P, Lamb P (P/P, n= 5). * represents $P<0.01$ vs PP.

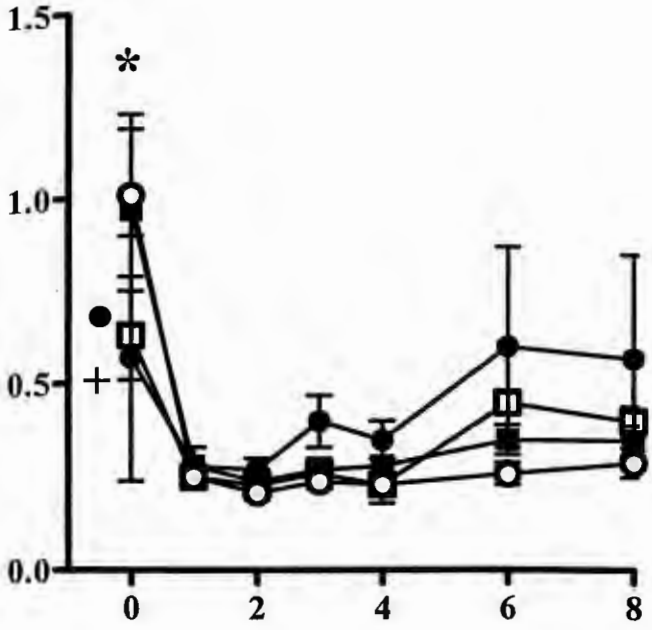

Fig. 2.4. Mean (± SEM) serum lysozyme concentrations in lambs from 0-8 wks of life. Vitamin E supplementation was given at 30 IU/kg BW/2 wks or Placebo, emulsified base. Maternal vitamin E (E) Lamb E (E/E, ■, n=10), Maternal E, Lamb placebo (P) (E/P, ○, n=11) Maternal P, Lamb E (P/E, ●, n=4), Maternal P, Lamb P (P/P, □, n=5). * represents $P<0.001$ E/E 0 vs E/E all weeks, E/P 0 vs E/P all weeks. + represents P/E 0 vs E/E 0 and E/P 0. ● represents P/P 0 vs P/P 1, 4.

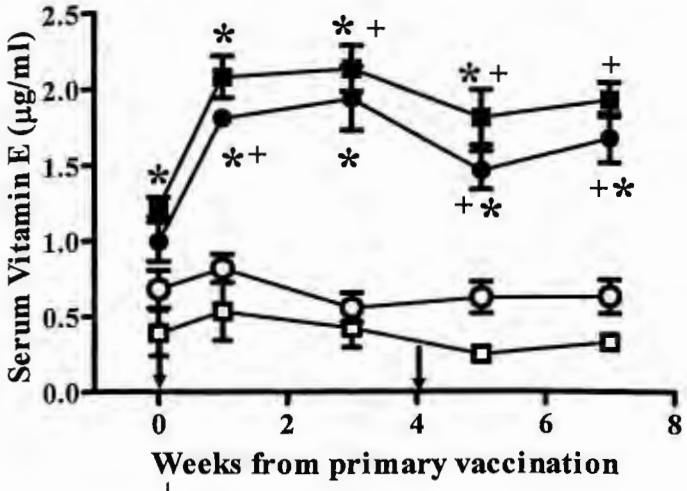

Fig. 2.5. Mean (± SEM) α-tocopherol concentrations in serum of lambs during vaccination period; Vitamin E supplementation was given at 30 IU/kg BW/2 wks or Placebo, emulsified base. Maternal vitamin E (E) Lamb E (E/E, ■, n=10), Maternal E, Lamb placebo (P) (E/P, ○, n=11) Maternal P, Lamb E (P/E, ●, n=4), Maternal P, Lamb P (P/P, □, n=5). ↓ indicates initial and booster vaccination against *Clostridium tetanus* and *clostridium perfringens*. * represents P≤0.001vs E/P. + represents *P*≤0.001 vs P/P.

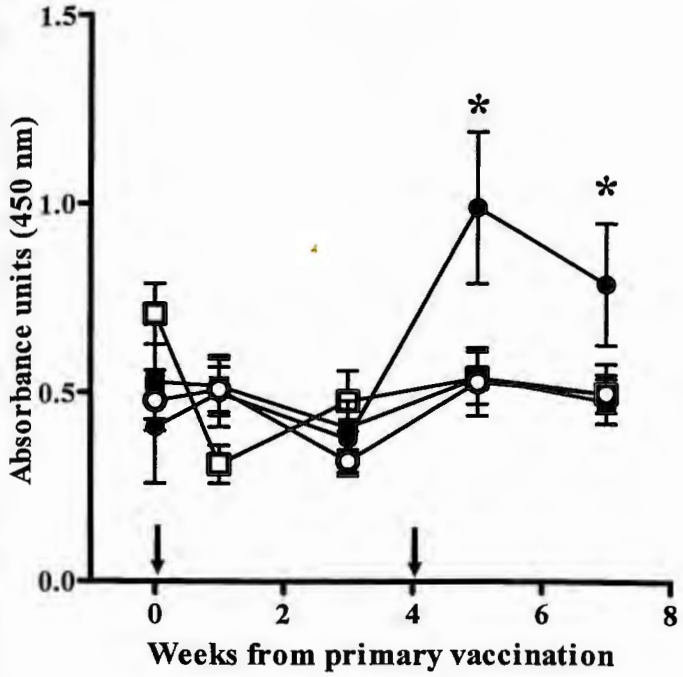

Fig. 2.6. Mean (± SEM) IgG concentrations against tetanus toxoid in serum of lambs during vaccination period; Vitamin E supplementation was given at 30 IU/kg BW/2 wks or Placebo, emulsified base. Maternal vitamin E (E) Lamb E (E/E, ■, n=10), Maternal E, Lamb placebo (P) (E/P, ○, n=11) Maternal P, Lamb E (P/E, ●, n=4), Maternal P, Lamb P (P/P, □, n=5). ↓ indicates initial and booster vaccination against *Clostridium tetanus* and *clostridium perfringens*. * represents $P \leq 0.001$ vs wk 0 & 3 within treatment.

Table 2.1. Results from proximate analysis and vitamin E analysis from composite feed samples collected over the study period[1].

Components	Grain Mix	Hay
DM, %	88.8	91.2
CP, % of DM	20.65	12.1
NDF, % of DM	29.7	59.2
ADF, % of DM	10.95	35.5
Calcium, % of DM	1.35	0.66
Phosphorus, % of DM	0.88	0.31
Magnesium, % of DM	0.38	0.23
Potassium, % of DM	0.98	2.01
NE_m, Mcal/Kg of DM	1.89	1.28
NE_g, Mcal/Kg of DM	1.26	0.71
Vitamin E, IU/Kg of DM[2]	46.3	50
α-Tocopherol, μg/g of DM[2]	42.14	45.47
Selenium, μg/g of DM[2]	1.13	0.03

[1]Proximate analysis conducted by Dairy One Cooperative, Inc. (Ithica, New York).
[2]Analyzed at the Diagnostic Center for Population and Animal Health (Michigan State University, Lansing, MI).

2.2. Mean (±SEM) of study variables in pregnant/lactating ewes supplemented with d-α-tocopherol (30 IU d-α-tocopherol /kg BW/2wks, n=10) or placebo (emulsified base, n=5)[1].

Variables	Placebo	Vitamin E
Serum/Plasma		
Vitamin E (µg/ml)[2,3]	2.14 ± 0.14	3.4 ± 0.2
IgG (mg/ml)[2,3]	16.65 ± 1.34	19.03 ± 1.25
IgGT (OD^{450})[3,4]	0.97 ± 0.13	0.91 ± 0.12
Lysozyme (µg/ml)[3]	0.28 ± 0.01	0.22 ± 0
GSH-Px[5,6] (nm NADPH/min/mg protein)	0.97 ± 0.17	0.88 ± 0.09
Se (ng/ml)[6]	215 ± 9.3[a]	197 ± 7.4
Colostrum[7]		
Vitamin E (µg/ml)	2.63 ± 0.53	13.36 ± 2.51[a]
IgG (mg/ml)	57.27 ± 7.3	57.28 ± 4.51
IgGT (OD^{450})	0.61 ± 0.12	0.56 ± 0.12
Lysozyme (µg/ml)	0.40 ± 0.14	1.18 ± 0.38
GSH-Px (nm NADPH/min/mg protein)	0.24 ± 0.15	0.22 ± 0.06
Se (ng/ml)	238 ± 19.7	184 ± 22.07

Within a row, means with a superscript small letter differ ($P < 0.05$).

[1]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[2]Treatment*week interaction is present ($P \leq 0.05$).

[3]Measured in serum.

[4]IgG against tetanus toxoid measured in absorbance at 450nm.

[5]Glutathione peroxidase.

[6]Measured in plasma.

[7]Variables were analyzed using Student's t test

Table 2.3. Effect of week (Mean ± SEM) on study variables in pregnant/lactating ewes[1, 2]

	Week of Supplementation [3]											
Variables	0	2	4	6	8	10	12	14	16	18	20	24
IgGT(OD^{450})[5,6]	0.59± 0.09^{ab}	0.45± 0.06^{bc}	1.11 ± 0.15^{de}	1.17± 0.11^{dfg}	1.42 ± 0.11^{fh}	1.10 ± 0.12^{degi}	0.92 ± 0.10^{eij}	0.83 ± 0.09^{jk}	0.99 ± 0.11^{degijk}	1.04 ± 0.11^{degij}	0.84 ± 0.11^{bjk}	0.71 ± 0.09^{bk}
Lysozyme[6] (µg/ml)	0.19± 0.01^{a}	0.21 ± 0.01^{abcdef}	0.2 ± 0.02^{ab}	0.22 ± 0.02^{abc}	0.22 ± 0.02^{abcd}	0.22 ± 0.02^{abcd}	0.25 ± 0.02^{bcd}	0.26 ± 0.02^{cde}	0.22 ± 0.02^{abcd}	0.23 ± 0.02^{abcd}	0.3 ± 0.02^{f}	0.31 ± 0.03^{ef}
GSH-Px[4,7] (nm of NADPH/min/mg of protein)	0.42± 0.06	0.66 ± 0.07^{ab}	0.8± 0.08^{abcd}	0.7 ± 0^{ace}	1.04 ± 0.05^{cdefg}	1.34 ± 0.2^{f}	1.03 ± 0.07^{dfg}	1.03 ± 0.06^{cdefg}	1.02 ± 0.1^{cdefg}	0.95 ± 0.05^{abcdeg}	1.02 ± 0.2^{abcdeg}	1.14 ± 0.25^{bdf}

[a-k] Within a row, means without a common superscript small letter differ ($P < 0.001$).

[1]Supplemented with d-α-tocopherol (30 IU d-α-tocopherol /kg BW/2wks, n=10) or placebo (emulsified base, n=5).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Vaccination against *Clostridium tetanus* and *Clostridium perfringens* at week 2 of supplementation.

[4]Glutathione peroxidase.

[5]IgG against tetanus toxoid measured in absorbance at 450nm.

[6]Measured in serum.

[7]Measured in plasma.

Table 2.4. Effect of age (Mean ± SEM) on study variables in pregnant/lactating ewes.[1,2]

Variables	Age	
	> 7 yrs	≤ 5 yrs
Vitamin E (μg/ml)[3]	2.82 ± 0.17	3.06 ± 0.12
IgG (mg/ml)[3]	18.68 ± 0.64	17.94 ± 0.38
IgGT (OD^{450})[3,4]	1.2 ± 0.05[a]	0.81 ± 0.04
Lysozyme (μg/ml)[3]	0.25 ± 0.01	0.22 ± 0.01
GSH-Px[5,6] (nm NADPH/min/mg protein)	0.97 ± 0.22	0.88 ± 0.09
Selenium (ng/ml)[6]	188 ± 3.93	208 ± 2.3[a]

Within a row, means with a superscript small letter differ ($P \leq 0.05$).

[1]Supplemented with d-α-tocopherol (30 IU d-α-tocopherol /kg BW/2wks, n=10) or placebo (emulsified base, n=5).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Measured in serum.

[4]IgG against tetanus toxoid measured in absorbance at 450nm.

[5]Glutathione peroxidase.

[6]Measured in plasma.

Table 2.5. Effect of treatment on Mean (±SEM) of study variables averaged over time in lambs from 0-8 wks of age supplemented with maternal and direct vitamin E (E/E, n=10), only maternal (E/P, n=11), only direct (P/E, n=4), neither maternal nor direct (P/P, n=5) [1,2]

Variables	P/P	P/E	E/E	E/P
Vitamin E[6] (μg/ml)	1.47±0.67	1.28±0.11	2.43±0.47[a]	2.24±0.42
IgG[6] (mg/ml)	12.47±1.41	9.75±2.17	12.28±1.32	11.06±1.07
IgGT[5,6] (OD^{450})	0.36±0.08	0.27±0.08	0.37±0.08	0.29±0.07
Lysozyme[3,6] (μg/ml)	0.35±0.05	0.43±0.15	0.39±0.06	0.36±0.05
GSH-Px[4,7] (nm of NADPH/min/mg of protein)	0.86±0.13	0.76±0.09	0.85±0.07	0.95±0.1
Selenium[7] (ng/ml)	100±7.6	107±7.4	97±3.5	99±4.2

Within a row, means with a superscript small letter differ ($P \leq 0.05$).

[1]Supplemented with d-α-tocopherol (E, 30 IU d-α-tocopherol /kg BW/2wks) or placebo (P, emulsified base).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Treatment*Time interaction is present.

[4]Glutathione peroxidase.

[5]IgG against tetanus toxoid measured in absorbance at 450nm.

[6]Measured in serum.

[7]Measured in plasma.

Table 2.6. Effect of week on Mean (± SEM) of study variables measured over the length of the study in 0-8 wk old lambs[1, 2]

Variables (Mean ± SEM)	Week of life						
	0	1	2	3	4	6	8
Vitamin E (µg/ml)	1.78 ±0.41	3.34 ± 0.39			1.92 ± 0.23		1.17 ± 0.12
IgG[5] (mg/ml)	0	18.38 ± 1.23	13.9 ± 0.93	10.9 ± 0.58[a]	9.05 ± 0.41[a]	9.65 ± 0.66[a]	7.33 ± 0.43
IgGT[4,5] (OD^{450})	0	0.54 ± 0.06	0.45 ± 0.05[a]	0.37 ± 0.05[a]	0.23 ± 0.03[b]	0.22 ± 0.03[bc]	0.14 ± 0.02[c]
GSH-Px[3,6] (nm of NADPH/min/mg of protein)	1.14 ± 0.05[b]	0.81 ± 0.08[a]	0.7 ± 0.04[a]	0.74 ± 0.03[a]	0.79 ± 0.04[ac]	0.98 ± 0.07[c]	0.97 ± 0.06[bc]
Selenium[6] (ng/ml)	71.1 ± 2.23	91.3± 2.7[a]	87 ± 2.04[a]	90.8 ± 2.37[ab]	97.7 ± 2.63[b]	122 ± 2.99	139 ± 3.57

[a-c]Within a row, means without a common superscript small letter differ ($P \leq 0.001$).

[1]Lambs were divided in to four groups based on maternal and direct vitamin E (E/E, n=10), only maternal (E/P, n=11), only direct (P/E, n=4), neither maternal nor direct (P/P, n=5). Supplementation was in the form of d-α-tocopherol (E, 30 IU d-α-tocopherol /kg BW/2wks) or placebo (P, emulsified base).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Glutathione peroxidase.

[4]IgG against tetanus toxoid measured in absorbance at 450nm.

[5]Measured in serum.

[6]Measured in plasma.

Table 2.7. Effect of treatment on Mean (±SEM) of study variables averaged over time in lambs during vaccination period supplemented with maternal and direct vitamin E (E/E, n=10), only maternal (E/P, n=11), only direct (P/E, n=4), neither maternal nor direct (P/P, n=5) [1,2]

Variables	P/P	P/E	E/E	E/P
Vitamin E[3,6] (µg/ml)	0.39±0.11	1.57±0.14	1.83±0.13	0.66±0.11
IgG[6] (mg/ml)	12.53±0.99	13.01±1	15.51±1.5	15.4±1.41
Lysozyme[6] (µg/ml)	0.34±0.10	0.41±0.10	0.35±0.05	0.24±0.03
GSH-Px[4,7] (nm of NADPH/min/mg of protein)	0.92±0.1	0.69±0.08	0.96±0.1	0.98±0.11
Selenium[7] (ng/ml)	150±8	160±15.3	165±6.5	154±7.2

[1]Supplemented with d-α-tocopherol (E, 30 IU d-α-tocopherol /kg BW/2wks) or placebo (P, emulsified base).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Treatment*time interaction is present ($P \leq 0.001$).

[4]Glutathione peroxidase.

[5]IgG against tetanus toxoid measured in absorbance at 450nm.

[6]Measured in serum.

[7]Measured in plasma.

Table 2.8. Effect of week on Mean (± SEM) of study variables measured over the length of the study in lambs during vaccination period[1, 2,3]

Variables (Mean ± SEM)	Weeks from primary vaccination				
	0	1	3	5	7
IgG[5] (mg/ml)	12.19 ±0.81[ab]	11.69 ± 0.78[a]	16.05 ± 0.92[b]	13.7 ± 0.7	19.6 ± 0.64
GSH-Px[4,6] (nm of NADPH/min/mg of protein)	1.18 ± 0.07[a]	1.12 ± 0.06[a]	1.01 ± 0.05[a]	0.74 ± 0.06	0.55 ± 0.05
Selenium[6] (ng/ml)	164 ± 4.11[a]	156 ± 4.55[ab]	161 ± 5.19[a]	152 ± 3.66[b]	155 ± 3.95[b]

[a-b]Within a row, means without a common superscript small letter differ ($P < 0.001$).
[1]Lambs were divided in to four groups based on maternal and direct vitamin E (E/E, n=10), only maternal (E/P, n=11), only direct (P/E, n=4), neither maternal nor direct (P/P, n=5). Supplementation was in the form of d-α-tocopherol (E, 30 IU d-α-tocopherol /kg BW/2wks) or placebo (P, emulsified base).
[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).
[3]Initial vaccination against *Clostridium tetanus and Clostridium perfringens* was at wk 0 and booster vaccination at wk 4 of the study.
[4]Glutathione peroxidase.
[5]Measured in serum.
[6]Measured in plasma.

Table 2.9. Effect of age on Mean (± SEM) of study variables averaged over time in lambs during vaccination period[1,2,3]

Variables	Age	
	>12 wks	< 12 wks
Vitamin E[6] (μg/ml)	1.23 ± 0.09	1.05 ± 0.07
IgG[6] (mg/ml)	14.58 ± 0.65	14.68 ± 0.54
Lysozyme[6] (μg/ml)	0.36 ±0.02	0.29 ± 0.01
IgGT[5,6] (OD^{450})	0.53 ± 0.03	0.5 ± 0.03
GSH-Px[4,7] (nm of NADPH/min/mg of protein)	0.87 ± 0.32	0.92 ± 0.37
Selenium[7] (ng/ml)	168 ± 2.4	151 ± 2.48[a]

Within a row, means with a superscript small letter differ ($P \leq 0.05$).

[1]Lambs were divided in to four groups based on maternal and direct vitamin E (E/E, n=10), only maternal (E/P, n=11), only direct (P/E, n=4), neither maternal nor direct (P/P, n=5). Supplementation was in the form of d-α-tocopherol (E, 30 IU d-α-tocopherol /kg BW/2wks) or placebo (P, emulsified base).

[2]Variables were analyzed using PROC MIXED in SAS (SAS Inst. Inc, Cary, NC).

[3]Initial vaccination against *Clostridium tetanus and Clostridium perfringens* was at wk 0 and booster vaccination at wk 4 of the study.

[4]Glutathione peroxidase.

[5]IgG against tetanus toxoid measured in absorbance at 450nm.

[6]Measured in serum.

[7]Measured in plasma.

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APPENDIX I

Standard Operating Procedure for the High Performance Liquid Chromatography (HPLC)

Standard Operating Procedure from Journal of Chromatography by Arnaud et al., 572:1-2, pg.103-16.

The vitamins are sensitive to white light, oxidation, and to drying. Work under the gold light, keep the extract under nitrogen, and resuspend the extract as soon as possible. The following parameters describe a HPLC system used to separate and quantitate □-tocopherol in samples extracted from serum and feed samples.

1) Place 1 ml of serum in a 16 x 125 labeled test tube. Samples other than 1 ml (0.5, etc.) can be run in the above manner, remember to take into account the appropriate sample weight. Add a volume of 0.9% NaCl to equal 1 ml to all samples that are less than 1 ml.
2) Add 1 ml of ethanol containing 0.01% BHT and 0.1% Ascorbic Acid.
3) Add 20uL of apocaroteneal internal standard.
4) Vortex for 5 minutes.
5) Add 1 ml HPLC grade hexane.
6) Vortex for 5 minutes.
7) Centrifuge at 3000rpm for 10 minutes.
8) Remove 0.5 ml of the top layer (hexane), dry using appropriate method.
9) Resuspend in 0.5 mL of mobile phase (70:20:10: Acetonitrile, Methylene Chloride, Methanol).

10) Transfer to HPLC vials and run on appropriate HPLC system.

-Mobile phase is 70:20:10 (v/v/v) acetonitrile: methylene chloride: methanol.

-Columns -guard column from Water's corp. Sentry guard column, C18, 3.5 μm

-analytical column: Water's corp. Symmetry C18, 3.5 μm, 4.6X75 mm column.

-Flow rate: use a isocratic at a flow rate of 1.2 ml/min.

-Detector: Photo diode array or variable wavelength UV detector is used. The wavelengths of interest are 292nm for alpha tocopherol, 265nm for cholecalciferol, 325nm for retinol and 450nm for beta carotene.

-Injection volume: 50 μl

APPENDIX II

Standard Operating Procedure for Lysoplate Assay

Lysozyme activity is measured by Lysoplate assay described according to Sotirov et al., (2005) with some modifications. Analyze the samples in duplicates.

- Preparation of culture media for Micrococcus luteus

  Peptone .....10 g

  Yeast extract ..... 5 g

  Sodium chloride ..... 5 g

  Glucose ..... 1 g

  Tryptone peptone ..... 11 g

  Agar ..... 12 g

  Distilled water .....1 L

  Autoclave at 121 C for 15 minutes and pour into petridishes.

- Preparation of standards: Prepare 1 mg/ml of the stock solution of Hen Egg White Lysozyme(HEWL) (Sigma) in distilled water and store at -20° C in aliquots.

1. Streak M.luteus culture (ATCC 27141) on the prepared media and incubate for 24 hr at 37° C.
2. Next, wash the bacterial culture with sodium phosphate buffer (Na_2HPo_4 & NaH_2Po_4, 0.7M pH-6.3) and mix it to form uniform suspension.
3. Adjust the optical density of the bacterial suspension to 1.8 and keep it in water bath at 60° C

4. Prepare 2% agarose in sodium phosphate buffer and keep it in water bath to attain 60°C temperature
5. Then, mix equal quantities of bacterial suspension and agarose in a container.
6. Mix it gently and pour 50 ml of agar + bacterial suspension in the grid petridishes.
7. Allow it to cool and keep it in refrigerator for 10 minutes.
8. Remove the petridishes from refrigerator and cut wells using 5 mm well cutter.
9. Prepare lysozyme standards running from 3.125 to 0.048 µg/ml in serial dilutions
10. Add 40 µl of standards and serum samples (directly without any dilution) or colostrum samples (1:10 dilution) into the wells in duplicates.
11. Incubate the loaded plates at 37° C for 24 hr.
12. After the incubation, the diameters of the clear, lytic zones formed around the samples are compared with the lytic zones of HEWL standards.
13. Results are expressed as µg of HEWL equivalent per ml.

APPENDIX III

STANDARD OPERATING PROCEDURE FOR IgG ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

Serum and Colostral IgG was measured using Sheep IgG kit (Bethyl labs, E130-101). The kit included Purified sheep IgG, Rabbit antisheep IgG coating antibody, Rabbit antisheep IgG HRP conjugated secondary antibody and TMB susbtrate.

Preparation of reagents and solutions

1. Prepare coating buffer by dissolving contents of one carbonate – bicarbonate capsule (Sigma-Aldrich; C-3041) in 100 ml distilled water to give 0.05 M and 9.6 pH.
2. To prepare washing/sample/conjugate diluents/blocking buffers, dissolve 1 packet of Tris buffer (Sigma-Aldrich; T-9039) containing 50mM Tris, 0.14M NaCl, 0.05% Tween 20, pH 8.0 in 1 L of distilled water.
3. Mix equal components of the TMB (Trimethyl benzidine) substrate just prior to use.
4. 2 M H_2SO_4 is used to terminate the reaction.

ELISA Procedure

1. Coat the wells of Nunc C bottom immunoplate 96 well plate (Fisher Scientific, 12-565-267) with a dilution of 1µl of capture antibody to 100µl of coating buffer.
2. Incubate the plate for 60 minutes.

3. Wash each well with washing buffer 3 times using ELISA plate washer (Biotek, ELX 405).
4. Add 200 µl of blocking buffer to each well
5. Incubate for 30 minutes and wash each well 3 times.
6. Dilute the standards in sample diluents according to the protocol.
7. Dilute the serum samples to 1:200,000 or 1:100,000 or 1:50,000 dilutions.
8. For colostrum samples, centrifuge at 24,400 g at 4° C. Remove the fat layer from top and collect the sample from bottom and dilute to 1:500,000 dilutions.
9. Incubate for 60 minutes at room temperature and wash each well 5 times.
10. Dilute HRP conjugated rabbit antisheep IgG in conjugate diluent to 1:100,000 dilutions and add 100ul/well.
11. Incubate for 60 minutes at room temperature and wash each well 5 times.
12. Mix equal volumes of TMB substrate just before adding to the wells and transfer 100ul/well.
13. Incubate for 15 minutes and 2M H_2SO_4 to terminate the reaction.
14. Read the plate at 450nm wavelength using microplate reader.
15. Subtract the blank reading from each value.
16. Create a standard curve using Gen 5 software and calculate the concentrations of the samples from the standard curve and the results are reported in mg/ml.

APPENDIX IV

STANDARD OPERATING PROCEDURE FOR IgG AGAINST TETANUS TOXOID ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

IgG against tetanus toxoid were measured according to the protocol described by Rosskopf et al. (2005) with modifications.

Reagents needed:

Tetanus toxoid (150 Lf/ml) (Colorado serum company, 61415)

Rabbit antisheep IgG HRP conjugated antibody (Bethyl labs, A130-101P)

Wash buffer, blocking buffer, sample diluents and conjugate diluents: Phosphate buffered saline + 0.05% Tween 20

Carbonate bicarbonate buffer (Sigma-Aldrich; C-3041)

1.25 M H_2SO_4

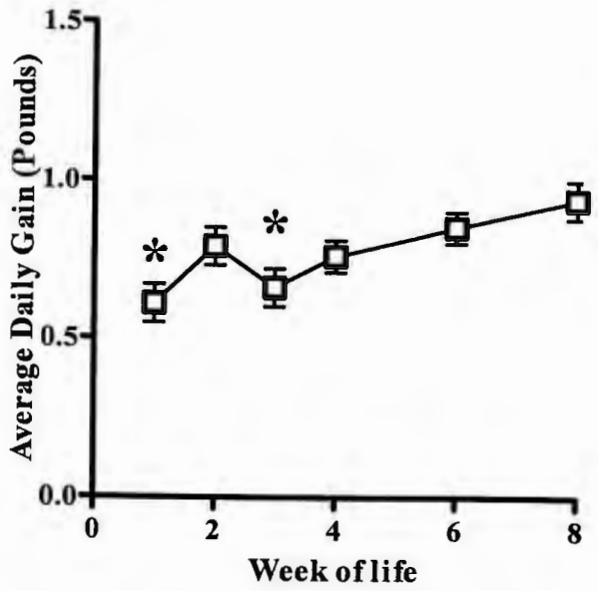
TMB susbtrate (Bethyl labs, E102)

ELISA procedure

1. Coat the wells of 96 well plate (Costar, 3590, flat bottom, high binding, nonsterile) with 0.2 Lf/ml of Tetanus toxoid in 0.05M carbonate buffer, pH 9.6, 100ul/well.
2. Incubate overnight at 4° C and wash the plate with washing buffer 3 times using plate washer (Biotek, ELX 405).
3. Add 250ul/well of blocking buffer and incubate for 60 minutes
4. Cover the plate with plate sealer and shake at 150rpm, 37° C for 1hr, followed by washing step 3 times.

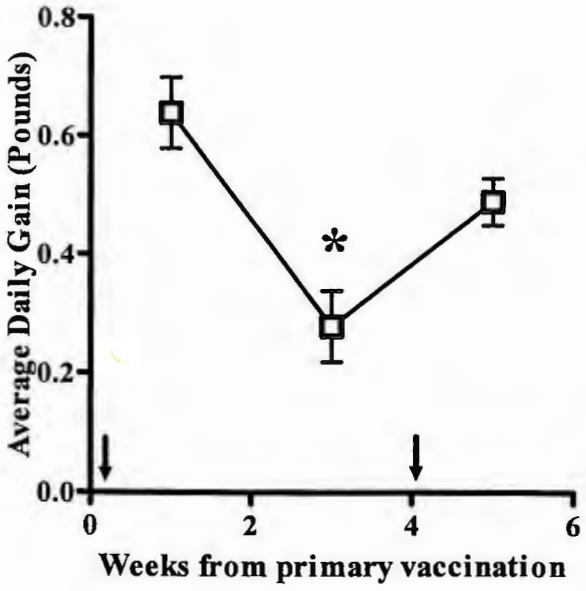
5. Dilute the serum samples to 1:2000 or 1:1000 dilutions in sample diluent.
6. For colostrum samples, centrifuge at 24,400g at 4° C for 20 minutes. Remove the fat layer from top and collect the sample from bottom and dilute to 1:10,000 dilutions.
7. Add the samples in duplicates.
8. Cover the plate with plate sealer and shake at 150rpm, 37° C for 1hr, followed by washing step 3 times.
9. Dilute HRP conjugated rabbit antisheep IgG in conjugate diluent to 1:20,000 dilutions and add 100ul/well.
10. Cover the plate with plate sealer and shake at 150rpm, 37° C for 1hr, followed by washing step 3 times.
11. Mix equal volumes of TMB substrate just before adding to the wells and transfer 100ul/well.
12. Incubate for 10 minutes and 1.25 M H_2SO_4 to terminate the reaction.
13. Read the plate at 450nm wavelength using microplate reader.
14. Subtract the blank reading from each value.
15. Record the readings in absorbance units.

APPENDIX V



Effect of week on Average Daily Gain (Mean ± SEM) of lambs from birth to 8 weeks of life. * represents $P \leq 0.001$ vs wk 6 & 8.

APPENDIX VI



Effect of week on Average Daily Gain (Mean ± SEM) of lambs vaccinated against *Clostridium tetanus* and *Clostridium perfringens*. * represents $P \leq 0.001$ vs wk 1 & 5.

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