Effect of excess degradable intake protein on ovarian steroids, oviductal proteins and early embryonic development in ewes

by Jie Weng

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science
Montana State University

Abstract:
The objective of this study was to determine if feeding a diet high in degradable intake protein during the estrous cycle alters blood urea nitrogen (BUN), ovarian steroids, oviductal proteins, or early embryonic development in sheep. Ewes were fed either a control (C) diet composed of mixed-grass hay that provided 100% of the NRC protein requirement for maintenance or the C diet plus a protein supplement (high protein; HP) which provides 200% of maintenance. Estrous cycles were synchronized with intravaginal sponges containing a progestogen. One half of the ewes on each diet had. their oviducts and uterine horns removed on either Day 2 or 4 after estrus and breeding to fertile rams. Jugular blood samples (10 ml) were collected daily from each ewe beginning at Day 2 of the synchronized estrous cycle and continuing until surgery. Right and left ampulla (AMP), isthmus (IST), and uterine horn (UTH) segments were flushed with Delbecco's PBS. Flushings were flash frozen in liquid N2. Protein in flushings was assayed using the BCA method. Serum samples were assayed for BUN, progesterone, and estradiol-17 β. Blood urea nitrogen concentrations were higher ($P < .05$) in HP ewes than C ewes during the synchronized cycle. Progesterone concentrations during the synchronized cycle did not differ ($P > .10$) between ewes on the HP and C diets. Estradiol-17(3 concentrations were higher ($P < .05$) in C ewes than in HP ewes during the periovulatory period. Feeding of the HP diet during the first 4 days of the next cycle did not affect ($P > .10$) AMP, IST, or UTH protein contents or concentrations but reduced ($P < .05$) -estradiol-17β concentrations, increased ($P < .05$) the rate of progesterone secretion between Days 2 and 4 after breeding, and delayed ($P < .05$) the rate of passage of early embryos through the oviducts. Feeding mature ewes excess degradable intake protein during a synchronized estrous cycle and during the first 4 days after breeding may contribute to embryonic loss by altering the ovarian steroid milieu after ovulation. Altering the steroid environment may delay embryo passage through the oviduct and result in a temporal asynchronization between the embryo and uterus. This in turn may affect the process of maternal recognition of pregnancy and result in embryonic death.

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ABSTRACT

The objective of this study was to determine if feeding a diet high in degradable intake protein during the estrous cycle alters blood urea nitrogen (BUN), ovarian steroids, oviductal proteins, or early embryonic development in sheep. Ewes were fed either a control (C) diet composed of mixed-grass hay that provided 100% of the NRC protein requirement for maintenance or the C diet plus a protein supplement (high protein; HP) which provides 200% of maintenance. Estrous cycles were synchronized with intravaginal sponges containing a progestogen. One half of the ewes on each diet had their oviducts and uterine horns removed on either Day 2 or 4 after estrus and breeding to fertile rams. Jugular blood samples (10 ml) were collected daily from each ewe beginning at Day 2 of the synchronized estrous cycle and continuing until surgery. Right and left ampulla (AMP), isthmus (IST), and uterine horn (UTH) segments were flushed with Delbecco's PBS. Flushings were flash frozen in liquid N_2. Protein in flushings was assayed using the BCA method. Serum samples were assayed for BUN, progesterone, and estradiol-17β. Blood urea nitrogen concentrations were higher ($P < .05$) in HP ewes than C ewes during the synchronized cycle. Progesterone concentrations during the synchronized cycle did not differ ($P > .10$) between ewes on the HP and C diets. Estradiol-17β concentrations were higher ($P < .05$) in C ewes than in HP ewes during the periovulatory period. Feeding of the HP diet during the first 4 days of the next cycle did not affect ($P > .10$) AMP, IST, or UTH protein contents or concentrations but reduced ($P < .05$) estradiol-17β concentrations, increased ($P < .05$) the rate of progesterone secretion between Days 2 and 4 after breeding, and delayed ($P < .05$) the rate of passage of early embryos through the oviducts. Feeding mature ewes excess degradable intake protein during a synchronized estrous cycle and during the first 4 days after breeding may contribute to embryonic loss by altering the ovarian steroid milieu after ovulation. Altering the steroid environment may delay embryo passage through the oviduct and result in a temporal asynchronization between the embryo and uterus. This in turn may affect the process of maternal recognition of pregnancy and result in embryonic death.

INTRODUCTION

The oviduct is an essential structure of the female reproductive tract. It is the site for ova transport, sperm capacitation, fertilization, and transport and development of the early embryo. After fertilization, the newly formed embryo is transported through the isthmus to the uterus. During transport the embryo undergoes its first cleavage divisions. The period during which the embryo transits the oviduct and enters the uterus lasts about 3 days for the ewe, 3.5 days for the cow, 2 days for the sow (Bearden and Fuquay, 1984a). By the time the embryo reaches the uterus, it is a blastocyst. The successful completion of these early divisions is essential to the survival of the embryo. The oviduct provides the site and the medium for these crucial steps.

Many factors affect reproductive efficiency of domestic ruminants. Dietary nutrients and their influence are among the most important of these factors. Nutrition can affect many reproductive processes in ruminants. For example, feeding ruminants a high plane of nutrition accelerates age at puberty, decreases postpartum interval, and alters placental size. On the other hand, feeding a low plane of nutrition has opposite effects. The mechanisms by

which nutrition changes reproductive processes are not well understood or even known. There is the possibility that changing the nutritional intake of ruminant females alters very early events of the reproductive cycle. These events occur in the oviduct. Thus, the oviduct may be a target for the effect of nutrition on reproduction.

Specifically, this thesis examines the hypothesis that the effect of nutrition on reproduction may be mediated by the oviductal environment in which embryos grow immediately after fertilization. An understanding of the role of nutrition in altering oviductal function may result in the discovery of novel mechanisms whereby specific nutrients alter reproductive efficiency in mammals. This in turn would allow us to develop techniques to optimize embryonic survival, fetal development, and birth weight in ruminants.

The present review focuses upon: a) the physiology of reproduction of ewe with emphasis on the physiology and histomorphology of the oviduct, b) the endocrinological control of oviductal function, c) the role of oviductal secretion in embryo development, and d) the influence of nutritional plane and/or dietary nutrients on reproductive efficiency.

REVIEW OF LITERATURE

Functional Anatomy and Physiology of the Ewe Oviduct

The oviducts are a pair of convoluted tubes extending from near the ovaries to and connecting the uterine horns. Oviducts of ewes are approximately 15 to 19 cm long and are divided into three functional segments: 1) the infundibulum, a funnel-shaped opening near the ovary, 2) the ampulla, or middle segment, and, 3) the isthmus, the narrow proximal portion of oviduct connecting the oviduct to the uterine lumen (Hafez, 1993a).

The infundibulum, which includes the fimbria, is that portion of the tube adjacent to the ovary. This region is responsible for guiding ovulated eggs into the opening of the middle segment or ampulla of the oviduct. The ampulla is from 3 to 5 mm in diameter and accounts for about half of the oviductal length. It is thin-walled with many easily distensible mucosal folds (Ellington, 1991). Once the egg is deposited in the ampulla, it is transported toward the uterus by two distinct methods. Wavelike contractions of the muscularis layer push the egg along the lumen, while the ciliary action of the epithelial cells also moves the

egg. Not all the epithelial cells are ciliated, however. The non-ciliated cells are secretory in nature and provide the oviductal fluid found in the lumen. This medium provides for fertilization, nourishment of the egg, and many other processes that will be discussed later in this review.

The ampullary-isthmic junction in ewes is difficult to locate anatomically. The isthmus contains fewer mucosal folds and a much narrower lumen than the ampulla, and it delays the ovum several hours during transport. Fertilization occurs at this junction. Sperm arrive at the site of fertilization through the isthmus, moving in much the same way as the egg. The movement of sperm is aided by flagellum (Hafez, 1993b).

The isthmus is thick walled and smaller than the ampulla, being 0.5 to 1 mm in diameter. Sperm capacitation and molecular changes on the egg occur in this region of the oviduct (Hafez, 1993a). The isthmus joins the uterine horn at the utero-tubal junction.

Functional Histology

Histologically, the oviduct is divided into three cell layers. The outer layer, basically connective tissue, is

the tunica serosa. The middle layer is the tunica muscularis, which includes both circular and longitudinal smooth muscle layers. The longitudinal muscle layer is closer to the lumen compared to the circular muscle layer. The innermost layer, made up of epithelial cells, is known as the tunica mucosa (Bearden and Fuquay, 1984b).

The outer serosal layer and the middle muscularis layers are continuous through the oviduct and perform the same functions in both regions. The serosa is a protective layer of connective tissue that helps keep the outer surface of the oviduct moist. The function of the muscularis layer is to move the contents of the oviduct by contraction.

The mucosa consists of a single layer of columnar epithelial cells containing ciliated and secretory cells. The total number of epithelial cells is greatest in the ampulla, because the lining is highly folded creating greater surface area (Lewis, 1990).

The secretory cells of the oviductal mucosa are nonciliated cells that have numerous long, slender microvilli and contain secretory granules. The size and number of secretory granules seen in these cells vary widely among individuals and with the phase of the estrous cycle (Hafez, 1993a). Secretory cell height reaches a

maximum at estrus. After ovulation, many granules are released and the cell height is reduced to a minimum during mid-cycle. These changes suggest that changes in ovarian steroids associated with estrus induce specific histological changes in the secretory cells.

The ciliated cells of the oviductal mucosa have slender motile cilia that extend into the lumen. Control of ciliary activity and numbers by systemic hormone levels has been described in several species, with increased numbers and motility seen near ovulation (Hunter, 1988).

The percentage of ciliated cells decreases in the ampulla toward the isthmus and reaches a maximum in the fimbriae and infundibulum. In the ampulla, cilia currents are barely detectable. In the isthmus, cilia tend to maintain a rapid and strong current directed towards the utero-tubal junction, presumably to facilitate embryo movement into the uterus at the 8- to 16-cell stages. The activity of cilia keeps oviductal eggs in constant rotation. This activity is thought to be essential for bringing the egg and sperm together (fertilization) and preventing oviductal implantation.

Oviduct Vasculature and Lymphatic Vessels

The vasculature of the oviduct is derived from the uterine and ovarian arteries. During estrus, blood flow to the oviduct increases (Weeth and Herman, 1952), presumably because of a change in ovarian estrogen which enhances the secretory activity of the tubal mucosa. Lymphatic vessels, which are more prevalent in the isthmus, dilate in the follicular phase, adding to the edema seen at estrus (Ellington, 1991).

Oviduct Innervation

The degree of innervation of the oviduct varies in the different muscle layers and in different regions of the oviduct (Hafez, 1993a). The ampullary and infundibular regions of the oviduct have limited innervation; whereas, the well developed circular muscle layer of the isthmus and the ampullary-isthmus junction contain rich adrenergic innervation where adrenergic nerve terminals are in close contact with individual smooth muscle cells (El-Banna and Hafez, 1970; Isla et al., 1989). In most species the isthmus contains mostly alpha-adrenergic receptors, so norepinephrine causes intense periovulatory contraction of the isthmus as a physiologic sphincter, which may be

important for regulating egg transport (Brundin, 1969).

Muscularis Activity

The oviductal musculature undergoes various types of complex contractions. In general the ampulla is less active than the isthmus. Oviductal muscularis activities are stimulated by contractions of two major membranes that are attached to the fimbriae, ampulla, and ovary: the mesosalpinx and the mesotubarium superius, which contain smooth musculature. Contractions usually proceed in an abovarian direction, adovarian contractions occur less frequently (Boling and Job, 1965). The frequency of contractions varies with the phase of the estrous cycle. Bennett et al. (1988) found that frequencies and amplitudes of contractions increased 3 to 5 days before estrus, resulting from segmental activity of the circular muscularis layer. These contractions decrease the internal isthmic diameter. Greatest muscle contractility is seen at estrus, especially at the utero-tubal junction in cows (Ruckebush and Bayard, 1975).

The varying patterns of oviductal contraction may be associated with cyclic changes in glycogen content of oviductal musculature. Glycogen in the oviduct is more

abundant in the inner circular musculature than in the outer longitudinal musculature.

Boling and Job (1965) found that the oviductal muscular activity in the rat increased when estrogen was withdrawn, but the pattern of contractility does not resemble the normal pattern as closely as that induced by progesterone. Whether this is the case in ewes is not known because there is no significant increase of progesterone during this phase of the estrous cycle in ewes.

Prostaglandin Activity

It has been suggested that in sheep, prostaglandin (PG)$F_{2\alpha}$ causes tubal contractions and PGE causes tubal relaxation (Harper, 1988). Both hormones increase tonus of the proximal part of the oviduct and cause relaxation in the remainder of the oviduct. However, PGE_3 relaxes the whole oviduct (Hafez, 1993a). On the other hand, $PGF_{2\alpha}$ stimulates contractions of the oviduct, aids sperm transport in female, and causes contraction of blood vessels.

Oviduct Fluid and Its Secretion

The fluid found in the oviductal lumen provides a suitable environment for fertilization and cleavage of fertilized eggs (Hafez, 1993a). The volume of oviductal fluid varies during the estrous cycle. The volume is low during the luteal phase of the cycle and begins to rise at the onset of estrus. Maximum amounts of fluid are secreted on the day after the onset of estrus, and the quantity thereafter declines rapidly to luteal phase levels (Perkins et al., 1965). The rate of accumulation of oviductal fluid is regulated by ovarian steroid hormones.

The directional movement of oviductal secretions may contribute to ovum transport to the uterus. The direction of flow of oviductal fluid is toward the ovary, because the isthmus blocks the flow of fluids into the uterus. In sheep, most of the oviductal secretions pass out of the oviductal ostium early in the estrous cycle. On day 4, however, when ova usually enter the uterus, fluid flow through the uterotubal junction increases remarkably.

Several important reproductive events occur in oviductal fluid. These include: 1) final maturation of the oocyte, 2) sperm capacitation, 3) fertilization, and 4) early embryonic development. Oviductal fluid is a

combination of blood transudate and secretory products of the granules from the secretory cells of the oviductal epithelium (Oliphant et al., 1984a and b).

A chemical analysis of the fluid from the sheep oviduct indicates that estrogen increases the secretion of potassium, bicarbonate, and lactate; whereas, progesterone decreases lactate secretion. Lactate could serve as a substrate for sperm metabolism (Restall and Wales, 1966a). Oviductal fluids harvested at different stages of the estrous cycle uniformly depress the respiratory activity of ram spermatozoa (Restall and Wales, 1966b). Cyclical changes occur in the pH of the oviduct in sheep. The lowest values occur during diestrus (6 to 6.4), the highest during estrus and metestrus (6.8 to 7.0) (Hadek, 1953).

Secretion of oviductal fluid by the epithelial cells provides the media for all of the events of reproduction that take place in the oviduct. By actively transporting serum components into the lumen, the secretory cells create a fluid medium with most of the necessary elements for cell survival. Proteins that do not originate in the serum make up the remaining portion of the oviductal fluid.

Oviductal Proteins

Fluid from the oviduct contains proteins from serum and proteins produced by the secretary cells of the oviductal lining (Leese, 1988; Brackett and Mastrioanni, 1974). Features of regional specificity and temporal release due to estrogen stimulation have been identified for protein components that are common to oviductal fluid and serum. Two types of glycoproteins are secreted by the tubal epithelium. One type is secreted throughout the cycle and the other is only produced in the peri-ovulatory phase. These proteins have been the subject of studies in a wide range of species. Oviduct specific glycoproteins have been found in all mammmals studied, from laboratory rats to humans (Abe and Abe, 1993 and Verhage et al., 1988).

In 1984, Sutton et al. performed an experiment to identify protein levels of sheep during the estrous cycle. Using a indwelling catheter technique they determined that protein concentration of the oviductal fluid increased 2- to 4-fold during the estrous cycle compared to non-cycling ewes.

Ellington et al. (1993) described experiments that involved culturing oviductal epithelial cells and analyzing the resulting protein secretions. Not only did they find oviduct-specific proteins, but they found that the types of proteins secreted depended upon whether the cells were in

contact, directly or indirectly, with sperm cells. The secretory cells changed the type of proteins they produced dependent on the environment. Cultured cells in contact with the sperm produced proteins that were not found in control cultures without sperm. This change in output could be an answer as to how sperm are capacitated in the oviduct.

An estrogen-dependent glycoprotein is produced by the epithelium of the ewe oviduct during the time the egg or fertilized ova are transported through the tube. Murray (1993) showed that proteins are secreted in response to hormonal control and are secreted in a region specific manner in the oviduct. She found that a 90,000 to 92,000 MW glycoprotein was secreted in response to estrogen in the ampulla, but not the isthmus, of cycling ewes on days 0 to 6 and Day 16 of the estrous cycle.

Many studies have shown a relationship between the improved survival of embryo transplants that are cultured with oviduct cells or fluids. Gandolfi et al. (1989b) discussed the role of the oviduct and its secretions on embryonic development. They concluded that oviducts improved the survivability of the embryos in vitro, and that some oviductal secretions may be essential for embryonic survival.

Role of Oviductal Secretion in Embryo Development

The oviduct provides a unique environment for sustaining embryonic development. It is known that the oviductal environment exerts a functional role in early embryonic development (Bavister, 1988). In particular, oviductal proteins are associated with early embryonic development in sheep (Gandolfi et al., 1989b; Murray, 1994). Most of these proteins are derived directly from the serum and are primarily serum albumin and immunoglobulins (Leese, 1988). The passage of proteins from the serum into the oviductal lumen is thought to be a case of selective transudation (Oliphant et al., 1978). Endocytosis plays a major role in this selective transudation process (Parr et al., 1988). The other source of oviductal proteins, primarily, high molecular weight glycoproteins, is that secreted by the oviductal epithelial cells in a wide variety of species (rabbit, Barr and Oliphant, 1981; sheep, Sutton et al., 1984; mouse, Kapur and Johnson, 1985; baboon, Fazleabas and Verhage, 1986; human, Verhage et al., 1988). The combined findings from biochemical and culture studies demonstrate that epithelial cells of the oviduct secrete proteins which are glycosylated (Sutton et al., 1985; Robitalle et al., 1988) and that these proteins

selectively interact with the embryo (Kapur and Johnson, 1986; Robitalle et al., 1988).

Oviductal glycoproteins are found associated with the zona pellucida, perivitelline space, and plasma membranes of blastomeres (Gandolfi et al., 1989a and b; Murray, 1993; Buhi et al., 1993). Gandolfi et al. (1989b) reported that the period of time during which the early embryo bound oviductal glycoproteins was reduced in vitro compared to that for in vivo embryos. They suggested that this might be due to lower secretion rates of these proteins by oviductal cells in vitro. Gandolfi et al. (1989a) found that in the sheep a protein specifically secreted by the epithelium at the time of embryonic passage through the oviduct is bound to the zona and incorporated into the cytoplasm. These findings demonstrate the possibility that other oviduct proteins may be translocated to the embryo and that the oviduct and embryo have a close biochemical relationship.

Effect of Nutrition

Adequate nutrients are required for many functions by animals, and the quantity required increase during times of increased production. Thus, nutritional needs are greater and more critical for many aspects of production, such

maintaining reproduction, lactation, and growth. Influences of nutrition on reproductive functions have been recognized for many years. However, underlying mechanisms are complex and in many cases are not well understood. Effects of early nutrition that influence the outcome of reproductive events are becoming a major research area (Lucas, 1992). Thus, it is important to understand the mechanisms when alterations in the supply of nutrients evoke changes in reproductive performance.

Early embryonic growth patterns may be related to nutritional status of dams (Robinson, 1990; Ashworth, 1994). During early embryonic and fetal development in ewes, dietary nutrient changes can affect ovulation rate and the size, vigor, and viability of the newborn (Robinson, 1996). The majority of studies about nutritional effects on embryo survival do not distinguish between fertilization failure and embryonic morality. Some studies have shown that when ewes were "flushed" (increase in nutrient intake) they were slower to conceive (Tassell, 1967a and b) and that ova from ewes on a high plane of nutrition have lower fertilization rates than those from ewes fed a low plane of nutrition (Lamond, 1970).

High levels of degradable intake protein in the diets of domestic ruminants have been associated with increased

fertilization failure (Blanchard et al., 1990). Protein level of diets can affect reproduction through toxic effects of ammonia and its metabolites on gametes and/or the early embryo (Ferguson and Chalupa, 1989). Toxic by-products of nitrogen metabolism from the rumen may impair sperm, ova, or early embryo survival. After feeding excess degradable intake protein, elevations of ammonia can interfere with intermediary metabolism and influence blood concentrations of glucose, lactate, free fatty acids, urea, and metabolic hormones (Visek, 1984) and corpus luteum functions (Garwacki et al., 1979).

Embryonic mortality is a major source of reproductive losses in most species of animals. In sheep, there is evidence that feeding either a low (Edey, 1966; Cumming et al., 1975; Hamra and Bryant, 1982) or a high (Hamra and Bryant, 1982; Parr et al., 1987) plane of nutrition during early pregnancy is detrimental to embryo survival. In a review of nutritional influences on embryo survival in cattle, sheep, and pigs, Robinson (1986) concluded that an extended period of under nutrition is required to cause significant reductions in embryonic growth and survival.

By using embryo transfer methods, Mckelvey and Robinson (1986) and Mckelvey et al. (1988) found that ewes fed a low plane of nutrition had higher embryonic survival

rates than ewes fed a high plane of nutrition. Specifically, the results of their reciprocal embryo-transfer experiments imply that it is more important for embryo survival than an increase in the plane of nutrition occurs during the pre- and peri-ovulatory periods than during early pregnancy; indeed, a high plane of nutrition during early pregnancy appears to be detrimental.

The mechanisms involved in the reduction of embryo survival arising from high-plane feeding in early pregnancy may be its affect on corpus luteum function or the secretion of progesterone. One of the physiological roles of progesterone is the maintenance of pregnancy. An inverse relationship has been observed between plane of nutrition and circulating progesterone concentrations in ewes (Parr et al., 1982; Williams and Cumming, 1982; Mckelvey et al., 1988; Rhind et al., 1985). Thus, increased progesterone metabolism occurs as a result of increased hepatic mixed-function oxidase activity in well-fed animals. Parr et al. (1987) demonstrated that ewes fed a high-plane of nutrition after mating had reduced progesterone concentrations and showed an increase in embryonic mortality. It would appear that feeding a high-plane diet, through its stimulatory effects on both hepatic blood flow and the metabolic clearance rate of progesterone (Symonds and Prime, 1989),

decrease progesterone concentrations which in turn are related to embryo development and survival. Changes in circulating progesterone concentrations modify the production of either the trophoblastic proteins and/or the endometrial secretory proteins (Knight et al., 1974; Roberts et al., 1988; Ashworth and Bazer, 1989). Some of these proteins can transport water-soluble nutrients across the placenta (Roberts et al., 1986). Progesterone-induced secretory proteins from the trophoblast play a critical role in the bi-directional signaling between mother and conceptus that is essential for maternal recognition of pregnancy and embryonic survival (Bazer, 1989; Ashworth, 1992). Pregnancy depends on a specific sequence of concentrations of progesterone and estrogen, and embryonic mortality may be caused by excesses or inadequate amounts of either of these ovarian steroids (Wilmut et al., 1986; Archibong et al., 1987).

The proposed mechanisms involved in the effects of high protein diets on the fertility of dairy cows have been reported. Elrod and Butler (1993) found that feeding high levels of degradable intake protein were associated with a reduction in pH of the uterine environment caused by excessive ammonia production by the rumen. Ammonia and urea differentially affected endometrial ion transport (Elrod,

1992) and contributed to reduced embryonic survival. Urea is one metabolite of dietary protein that is formed from detoxification of NH_4 by the liver. The concentration of urea in the plasma or blood (PUN or BUN) is reflective of the quantity and degradability of the protein. Plasma urea nitrogen (PUN) concentrations have often been used as a correlate between dietary protein level and fertility. High concentrations of PUN are associated with increased rumen ammonia. High degradable intake protein diets cause excessive ammonia production in the rumen and conversion of ammonia to urea (Oltjen et al., 1972). At high ruminal ammonia concentrations, the capacity of the liver for urea synthesis is exceeded, ammonia would accumulate in the blood, and ammonia toxicity may result.

The hypothesis that excess rumen ammonia may affect early embryonic growth and development is illustrated by the following results of in vivo and in vitro embryo culture and embryo transfer experiments. Thompson et al. (1994) found that birth weights of lambs from embryos cultured in synthetic oviduct fluid (SOF) were greater than those from spontaneously ovulating ewes. For spontaneously ovulating animals, Ferguson and Chalupa (1989) have expressed concern at the low conception rates to first insemination in high-producing dairy cows. They suggested

that a major cause might be an excess of degradable intake protein leading to toxic effects of ammonia and its metabolites on gametes and/or embryos. Embryos cultured in SOF had higher concentrations of ammonia and induced high birth weights and dystocia in ewes (Thompson et al., 1994). The toxic effects of ammonia on cells (Visek, 1984), and specifically reproductive tissues (Stalheim and Gallagher, 1977), have been demonstrated. Elevated plasma ammonia (>100 μmol/l) induced higher occurrence of embryonic death in sheep (Bishonga et al., 1994).

STATEMENT OF THE PROBLEM

Dietary nutrients can affect many reproductive processes in ruminants. It is well known that excess degradable intake protein and high feeding levels affect early embryonic development and survivability. The mechanism of this effect may be related to the liver's ability to accommodate the challenge of excess NH_4 production, which in turn will affect reproductive process. The goal of our research is to investigate the relationship between high protein diets and reproductive changes, and influences of excess degradable intake protein on oviductal secretion and early embryonic development. The review of the literature indicates that the effects of excess degradable intake protein on embryonic survival may occur very early in embryonic development. Specifically, it may occur during the initial stages of development in the oviduct. The effect(s) may be carried over into later stages of development or cause a change in the ability of the embryo to synchronize its development with uterine changes necessary to sustain it. Presently, there are no data in the literature regarding the effect of protein nutrition on oviductal protein secretion or on the ovarian steroid environment during the first few days of embryonic

development in ruminants. Therefore, the objectives of this study were to determine if: 1) feeding mature ewes a diet high in degradable intake protein (HP) alters blood urea nitrogen and ovarian steroid concentrations during the estrous cycle or soon after fertilization, 2) oviductal protein concentrations or their patterns are altered in ewes fed a HP diet, and 3) early embryo development is altered by feeding ewes a HP diet.

It is our hypothesis that feeding HP diets reduces embryo survival through its influences on oviductal functions. The importance for this reproductive limitation is obvious if considered from the producer standpoint. Low rates of embryo survival of animals causes economic lose to the producer by reducing reproductive efficiency. By understanding the cause of poor reproductive performance, we may be able to change this effect and increase production efficiency. Furthermore, we may obtain information regarding a biologically important mechanism whereby the effect of a specific macronutrient influences reproductive processes.

MATERIALS AND METHODS

Animals

This study was performed between October and December of 1996. Thirty-one mature, western white-faced ewes, which were multiparous and 3- to 6- yr-old, were used. Ewes were housed at the Fort Ellis Sheep Research Station of Montana State University, Bozeman.

Estrous Synchronization

Medroxy-aceto-progesterone (MAP) intravaginal sponges were inserted into each ewe to synchronize estrous cycles. Sponges were removed 14 days after insertion. Ewes were observed for estrus with the aid of sterile epididectomized (teaser) rams beginning 36 hours after the removal of the sponges. Ewes were checked twice daily for 4 days. Only ewes that would stand to be mounted by rams were assigned to treatments. Day of estrus was defined as Day 0 of an estrous cycle.

Experimental Treatments

Diets

At the synchronized estrus each ewe was assigned randomly to receive either a maintenance diet (C; control) or the maintenance diet plus supplemental protein (HP; high protein). Ewes assigned to the control diet were fed mixed-grass hay that had a crude protein (CP) content of 0.123 kg. Ewes assigned to the HP diet were fed mixed-grass hay (CP = 0.123 kg) and a soybean supplement. The crude protein content of the soybean meal was 44%. The supplement was fed at a rate of 0.088 $kg.hd^{-1}.d^{-1}$. The total CP for HP diet was 0.211 $kg.hd^{-1}.d^{-1}$. This level of crude protein provided approximately 200% of the NRC requirement for maintenance (NRC, 1985) for these ewes. Water and mineralized salt were provided ad libitum. Ewes were placed into separate pens and fed hay and/or HP supplement beginning on Day 2 of the synchronized estrous cycle. Ewes were fed the supplement and given the hay ration in the morning of each day throughout the synchronized cycle. The number of ewes on each diet is given in Table 1. Composition of diets is given in Table 2.

Day of Estrous Cycle for Surgical Removal of Oviduct and Uterine Horns

On Day 16 of the synchronized estrous cycle, ewes on each diet were placed with teaser rams and observed for estrus twice daily. When a ewe was observed to display estrus she was mated to two different fertile rams. She was then assigned to undergo surgical removal of the oviducts and uterine horns on either Day 2 or 4 after estrus. The number of ewes on each diet that were assigned to each day of the estrous cycle is given in Table 1.

Table 1. Experimental design and numbers of ewe per treatment.

Day of Estrous Cycle	Diet		
	Control (C)	High Protein (HP)	Total
2	7	8	15
4	8	8	16
Total	15	16	31

Table 2. Chemical composition (% of DM) of diets.

Item	Mixed-grass hay[a]	Soybean meal[b]
Crude Protein	10.0	44.0
Crude Fat		0.5
Crude Fiber		7.0

[a]Chemical analysis determined by laboratory procedures.
[b]Chemical analysis provided by feed manufacturer (Westfeeds, Big Sky Division, Biiilings, MT)

Surgical Procedures

Thiopental Sodium (10 ml; 4 %) was injected into a jugular vein to anesthetize each ewe. A mid-ventral incision was used to exteriorize the ovaries, oviducts, and uterine horns. Side and number of ovulations were recorded. Oviducts were ligated at the utero-tubal junction, the isthmic-ampullary junction, and the ampullary-infundibular junction to insure that no fluid was lost and that no transfer of fluids or contents occurred between regions. Uterine horns were ligated at the external bifurcation of the uterus. The blood supplies to the oviducts and uterine horns were then ligated, and the oviducts and uterine horns removed. Immediately after removal, the oviduct and uterine tissues were transported to the laboratory in a pre-warmed Styrofoam container. After surgery, Sodium Pentobarbital (3ml; 35%) was injected into a jugular vein for euthanasia.

Blood Samples

Blood samples (10 ml) were collected daily from all ewes by jugular venipuncture each morning beginning on Day 2 of the synchronized estrous cycle until the day of surgery of the next cycle. Blood samples were allowed to clot at 25°C for 3 to 4 hours. They were then centrifuged at 1,285 *x g* for 20 minutes at 4°C. Serum from each sample was decanted into 12 x 75 mm plastic culture tubes and stored at -22°C for later assay of blood urea nitrogen (BUN), progesterone (P4), and estradiol-17β (E2).

Processing Oviduct and Uterine Tissue

Weight and length of each segment of oviduct and uterus were recorded in the laboratory. In vivo oviduct and uterine tissue secretions were collected from each ampulla (AMP), isthmus (IST), and uterine horn (UTH) by flushing each twice with 3, 1.5, and 10 mL of Delbecco's phosphate-buffered saline. Flushings from AMP, IST, and UTH ipsilateral to the ovulating ovary were searched microscopically for the presence of ova or embryos. Flushings of AMP, IST, and UTH from each side were then pooled. Pooled flushings were placed on ice and PMSF was added to a final concentration of 10 mM. Samples were flash

frozen in liquid N_2 and stored at -22°C for assay of protein content and concentration.

Assays

Blood Urea Nitrogen Assay

The concentration of blood urea nitrogen (BUN) is regulated by the metabolism of proteins and by the renal excretion. Blood urea nitrogen concentrations were measured by coupled enzyme reactions involving urease and glutamate dehydrogenase. One mL of BUN Endpoint reagent and 0.005 mL (5 μL) of serum was pipetted into each tube and incubated at 37°C for 5 minutes. Sample absorbance was read and recorded at 340 nm. Range of the standard curve was 0 to 40 mg/dL. The sensitivity of the assay was 5 mg/dL. Inter- and intra-assay CV's were less than 5%.

Bicinchoninic Acid (BCA-Protein) Assay

A detergent compatible formulation based on BCA for the colorimetric detection and quantification of total protein was used to assay oviductal and uterine protein

content. A fresh set of protein standards was prepared by diluting the 2.0 mg/mL BSA stock standard (Stock). Fifty parts of BCA Reagent A with 1 part of BCA Reagent B were mixed (BCA Working Reagent) just before the assay. Two hundred μL of each standard or AMP, IST, or UTH flushing was pipetted into appropriately labeled test tubes. Two mL of the working reagent was added to each tube. All tubes were incubated at 60°C for 30 minutes. All tubes were cooled to room temperature and the absorbance measured at 562 nm using water as a reference. The range of the standard curve was 0 to 40 μg/200μL. The sensitivity of the assay was 5 μg/200μL and the inter- and intra-assay CV were less than 5%. Concentration was calculated by multiplying the assay content by the volume of the flushing then dividing by the weight of the tissue segment.

Progesterone Assay

Progesterone was assayed by a solid-phase radioimmunoassay (RIA) with kits purchased from Diagnostic Products Corp. (Los Angeles, CA). Briefly the procedure was as followed. Four plain (uncoated) 12 x 75 mm polypropylene tubes were labeled in duplicates for total

counts and non-specific binding tubes. Fourteen coated Progesterone Ab-Coated Tubes A (maximum binding) and B through G were labeled in duplicate. Additional antibody-coated tubes were also prepared for samples in duplicate. One hundred μL of the zero calibrator A was pipetted into the NSB and A tubes and 100 μL of each of the calibrators B through G into correspondingly labeled tubes. One hundred μL of each sample was placed into the appropriately labeled tubes. One mL of ^{125}I-progesterone was added to all the tubes and the tubes were incubated overnight at room temperature. Each tube, except the total count tubes, was decanted thoroughly and counted for 1 minute in a gamma counter. The sensitivity of the assay was 0.02 ng/mL. The inter- and intra-assay CV were less than 12%.

Estrogen Assay

Estradiol-17β was quantified by a double antibody RIA using kits purchased from Diagnostic Products Corp. (DPC; Los Angeles, CA). Samples were extracted before each assay. For the extraction procedure 200 μL of serum or standards were pipetted into 16 x 100 mm borosilicate disposable culture tubes, and 75 μL of ^{125}I-E2 was pipetted into four

tubes to estimate recovery of the steroid. Two mL of ethyl acetate was added to each of the tubes. Samples were vortexed for 3 minutes then centrifuged at 600 *x g* at 4°C for 10 minutes. The supernatants were removed with pasteur pipettes and placed into 12 x 75 mm culture tubes labeled with the same numbers as the 16 x 100 mm tubes from which we removed the supernatants. Then the 12 x 75 culture tubes were dried in a warm (37°C) water bath with N_2. The extraction was repeated. Then 200 µL of 0.1% gelatin in 0.1 M Phosphate buffer saline was added to all the tubes. They were then incubated at 4°C overnight.

One hundred µL of the extracted sample was pipetted into another identically labeled tube. Thirty µL of E2 antibody from DPC was added to all tubes except the total count tubes (TCT), non-specific binding tubes (NSB), and buffer-blank tubes. Samples were incubated at room temperature for two hours. Seventy-five µL of ^{125}I-E2 (25,000 cpm) was added to all tubes which were then incubated at room temperature for 1 hour. One mL of Precipitating Solution was added to all tubes except the TCT tubes and incubated for 10 minutes at 4°C. After this incubation all of the tubes except the TCT tubes were centrifuged at 1,285 *x g* for 20 minutes at 4°C. The

supernatants were decanted, except the TCT tubes, and dried down on blotting paper. The samples were counted for 5 minutes in a gamma counter. The sensitivity of the assay was 0.16 pg/mL. The inter- and intra-assay CV were 15% and 10%, respectively.

Statistical Analyses

Synchronized Estrous Cycle Length

Estrous cycle length for ewes was analyzed by a one-way ANOVA using the GLM procedure of SAS (SAS, 1996). The model included only diet.

Blood Urea Nitrogen Concentrations During the Synchronized Estrous Cycle

Blood urea nitrogen concentrations throughout the synchronized estrous cycle of two ewes from each diet were evaluated visually. After this assessment it was decided that the statistical analyses would include samples obtain from ewes on Day 15 of the synchronized estrous cycle through Day 4 of the next estrous cycle. These data were analyzed with a split-plot ANOVA for a completely random

design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

Progesterone Concentrations During the Synchronized Estrous Cycle

Progesterone concentrations during the synchronized estrous cycle of ewes fed the control or HP diets were analyzed with a split-plot ANOVA for completely random design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

Estradiol-17β Concentrations During the End of the Synchronized Estrous Cycle and Beginning of the Next Cycle

Estradiol-17β Concentrations data from Day 17 of the synchronized cycle through Day 4 of the next cycle were analyzed with a split-plot ANOVA for completely random

design using the GLM procedure of SAS (SAS, 1996). The main plot included diet and the error term to test the effect of diet which was animal within diet. The sub-plot included day of cycle and the interaction of diet and day of cycle.

BUN, P4, and E2 Concentrations on Days 2 and 4 of the Estrous Cycle of Breeding

Blood urea nitrogen, P4, and E2 concentrations in blood samples taken from ewes on either Day 2 or 4 after estrus and mating to fertile rams were analyzed by an ANOVA using the GLM procedure of SAS (SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet, day of surgery, and their interaction. Within class correlation coefficients were generated among BUN, P4, and E2 concentrations using the CORR procedure of SAS (SAS, 1996).

Oviductal Protein Content and Concentration

Protein content and concentrations of the AMP, IST, and UTH were analyzed separately using ANOVA for a completely random design using the GLM procedure of SAS

(SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet, day of surgery, and their interaction.

Ovulation Rates and Embryo Development and Location Within the Reproductive Tract

Ovulation and embryo recovery rates, proportion of embryos damaged, and the proportion of embryos located in either the AMP, IST, or UTH were analyzed by separate contingency Chi-square tests. Cell stage of embryos was analyzed by ANOVA for a completely random design using the GLM procedure of SAS (SAS, 1996) with treatments arranged factorially (2 x 2). The model included the main effects of diet (control and high protein), day of surgery (Days 2 and 4), and their interaction.

RESULTS

Estrous Cycle Length

Estrous cycle lengths did not differ ($P > .10$) between ewes fed a high protein (HP) or control (C) diet, and were 17.4 ± .3 (mean ± se) and 17.3 ± .3 days, respectively.

Patterns of Blood Urea Nitrogen During the Synchronized Estrous Cycle

There was no interaction ($P > .10$) between diet and day of the estrous cycle for BUN concentrations from Day 15 of the synchronized cycle through Day 4 of the next estrous cycle (Figure 1). However, BUN concentrations were higher ($P < .05$) in ewes fed the HP diet than in ewes fed the C diet during the synchronized estrous cycle and the beginning of the next estrous cycle (Table 3).

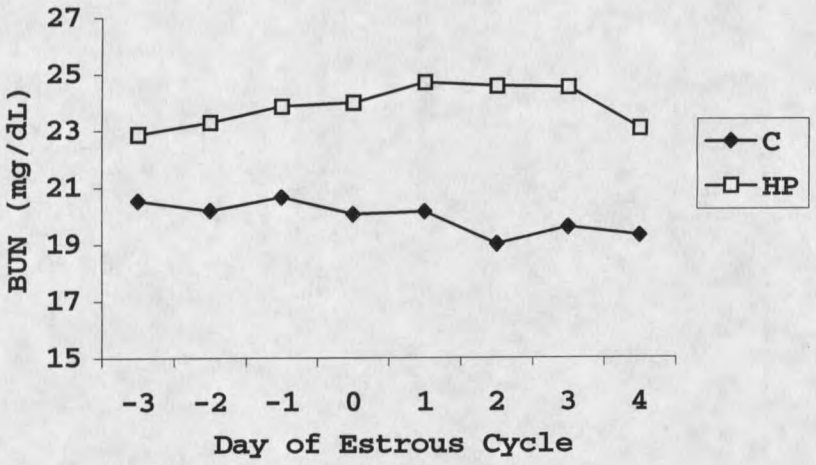

Figure 1. Blood urea nitrogen (BUN) concentrations from Day 15 of the synchronized estrous cycle through Day 4 of the next estrous cycle. Numbers of ewes through Day 2 for the control (C) and high protein (HP) diets were 15 and 16, respectively. Data for Days 3 and 4 represent 8 and 8 ewes from the C and HP diets, respectively.

Table 3. Least square means of BUN concentrations (mg/dL) for ewes fed either the control or high protein diet during the synchronized estrous cycle and the beginning of the next estrous cycle.

Item	Diet[a]		
	Control (15)	High Protein (16)	ANOVA
BUN (mg/dL)	19.0	24.0	P < .05

[a]SEM = 3.6.