EFFECT OF LINSEED MEAL ON THE REPRODUCTIVE AND DIGESTIVE TRACTS
IN SHEEP

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MASTER OF SCIENCE

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ABSTRACT


To evaluate the estrogenic potential of secoisolariciresinol diglycoside (SDG) found in linseed meal (LSM) on visceral organ mass, uterine mass, jejunal cellularity, jejunal vascularity, and angiogenic factor expression in the jejunum, 48 multiparous, ovariectomized ewes were used in a 3 x 4 factorial. Ewes were fed a diet containing 12.5% LSM for 0, 1, 7, or 14 d and implanted with estradiol 17-β (E2) for 0, 6, or 24 h before tissue collection. This study confirmed that LSM interacts with E2 to alter uterine and duodenal mass; that E2 is capable of altering liver, duodenal, and uterine mass; and that LSM is capable of altering liver and duodenal mass. In addition to altering organ mass, LSM interacted with E2, causing E2 to have no effect on cellular proliferation rate of the jejunum when LSM was fed for 1, 7, or 14 d, whereas cellular proliferation was increased in the duodenum in response to E2 when LSM was fed for 0 d. However, neither LSM nor E2 altered any measurement of vascularity. Expression of angiogenic factor mRNA was also examined. Implanting ewes with E2 for 6 h increased eNOS mRNA expression; whereas, LSM and E2 interacted to alter mRNA expression of its receptor, sGC, as well as VEGF’s 2 receptors, FLT and KDR. Although this study confirmed LSM’s ability to interact with E2, further research is needed to investigate its effects on intact cyclic animals as well as in pregnant animals and their offspring.
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me to pursue my goals. It is disheartening to consider all those who failed to achieve what
they set out to accomplish, not for want of talent, skill, or intelligence, but because they
lacked the encouragement or reassurances from others to persist in the pursuit of their
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<th>Definition</th>
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<tr>
<td>ADF</td>
<td>Acid detergent fiber</td>
</tr>
<tr>
<td>ANG-1</td>
<td>Angiopoietin-1</td>
</tr>
<tr>
<td>ANG-2</td>
<td>Angiopoietin-2</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBW</td>
<td>Empty body weight</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol-17β</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor 2 IIIc</td>
</tr>
<tr>
<td>FLT</td>
<td>VEGF receptor-1</td>
</tr>
<tr>
<td>FSM</td>
<td>Flaxseed meal</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>KDR</td>
<td>VEGF receptor-2</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>Meal</td>
<td>Megacalories</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
</tr>
<tr>
<td>NEₘ</td>
<td>Net energy of maintenance</td>
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X
NFE .......................................................... Nitrogen free extract
RNA .......................................................... Ribonucleic acid
SAS .......................................................... Statistical analysis system
SDG .......................................................... Secoisolariciresinol diglycoside
SEM .......................................................... Standard error of the mean
sGC .......................................................... Soluble guanylate cyclase
Tie-2 .......................................................... Angiopoietin receptor
T3 ............................................................ Triiodothyronine
T4 ............................................................ Thyroxine
VEGF ..................................................... Vascular endothelial growth factor
Wt ............................................................ Weight
CHAPTER 1
INTRODUCTION, LITERATURE REVIEW, AND STATEMENT
OF THE PROBLEM

Introduction

Flax (*Linum unisatissimum*) is an oilseed grown predominately in the upper Midwest of the United States and Canada. Flaxseed and flaxseed-derived products are often incorporated into livestock diets. There are also reports that health benefits of flaxseed consumption exist for humans. Linseed oil, the oil derived from flaxseed, contains a high proportion of the omega-3 fatty acid linolenic acid. The health benefits of consuming linolenic acid and omega-3 fatty acids, in general, are vast, well established, and continue to be elucidated.

In addition to omega-3 fatty acids, flaxseed also contains secoisolariciresinol diglycoside (SDG). This compound belongs to a class of chemicals called lignans which are termed phytoestrogens. Research indicates that SDG possesses estrogenic and antiestrogenic effects both in vitro and in vivo. In addition to effects from omega-3 fatty acids when consuming flaxseed, SDG may also cause physiological effects important in animal growth and metabolism, carcass composition, and reproduction as well as potential human implications such as antioxidant activity and tumor suppression.

As flaxseed and flaxseed-derived products become increasingly used as a feed source for livestock, it becomes increasingly important to fully understand the health, production, and economic consequences of feeding flaxseed to livestock. Much research has been done to investigate the effects of isoflavones found in soy on humans and animals. Relatively little data exist investigating the effects of lignans on livestock, and many of the
studies investigating the effects of flax or flax-derived products on livestock production focus on omega-3 fatty acids rather than the estrogenic lignan portion of flax. The use of flax as a livestock feed and as a health supplement for humans necessitates the need for continued research to fully understand flax and SDG’s biological effects as well as the mechanism through which it elicits these effects.

**Literature review**

**Flax as a Livestock Feedstuff**

Flaxseed is an oilseed crop grown predominately in North Dakota and Canada. It is composed of approximately 36% oil, 24% protein, and 32% carbohydrates (Collins et al., 2003). The oil in flaxseed is used extensively for the production of paint, varnish, lacquer, printing ink, linoleum flooring, and as a treatment for concrete surfaces (Dyck, 2001). The fiber is used predominately to make fine paper products such as quality stationary and cigarette paper. From the years 2000 to 2005, the United States produced an average of 12,437,833 bushels of flaxseed. North Dakota alone accounted for over 94% of total U.S. flaxseed production (USDA, 2005). Additionally, the utilization of flaxseed as an animal feed could provide both a readily available feed source in the upper Midwest as well as a valuable consumer of linseed meal (LSM) for flax producers.

The inclusion of flaxseed in animal diets is becoming widely practiced. The high protein content of flax and LSM in addition to the high fat content of flax makes both of them useful as supplements in a variety of production systems (Table I). Research indicates that the inclusion of as little as 5% flaxseed in swine diets increases the amount of omega-3 fatty acids deposited in the carcass (Cunnane et al., 1990; Romans et al., 1995). Drouillard et al. (2002 and 2004) also reported that feeding flax in beef cattle finishing
diets increased levels of omega-3 fatty acids and often increased carcass quality grades compared with cattle finished with other sources of dietary lipids. Similarly, they found that levels of omega-3 fatty acids increased in the tissue lipid portion of the carcass, specifically alpha-linolenic acid and eicosapentaenoic acid when animals were fed linseed oil, the oil component of flaxseed (Drouillard et al., 2002, 2004). In addition to shifting carcass fatty acid composition to a more desirable fatty acid profile, the inclusion of flaxseed in the diet was not accompanied with a depression in feed intake as observed when other sources of dietary lipid such as tallow are included in the diet. Furthermore, Drouillard et al. (2002) observed that the inclusion of ground flaxseed, linseed oil, or tallow in the diet of feedlot cattle reduced the number of animals requiring treatment for bovine respiratory disease. Among cattle that did require treatment for bovine respiratory disease, there was a reduction in the number of animals that needed to be retreated when ground flaxseed or linseed oil was fed. Maddock et al. (2006) compared flaxseed and LSM and observed improved gains, feed efficiency, yield grade, and marbling in beef heifers fed flaxseed compared with heifers fed LSM and that steaks from heifers fed flaxseed were tenderer than steaks from heifers fed LSM.

<table>
<thead>
<tr>
<th>Item</th>
<th>Flax</th>
<th>LSM</th>
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<tr>
<td>DM, %</td>
<td>93.6</td>
<td>91.2</td>
</tr>
<tr>
<td>Fiber, %</td>
<td>6.3</td>
<td>8.2</td>
</tr>
<tr>
<td>CP, %</td>
<td>25.1</td>
<td>42.2</td>
</tr>
<tr>
<td>Fat, %</td>
<td>38.9</td>
<td>6.1</td>
</tr>
<tr>
<td>NFE, %</td>
<td>25.9</td>
<td>37.8</td>
</tr>
<tr>
<td>Mineral, %</td>
<td>3.8</td>
<td>5.6</td>
</tr>
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</table>

1Morrison (1946).
In addition to flaxseed’s effects on animal performance and carcass characteristics, the dairy industry has also shown an interest in using flaxseed as an ingredient in dairy rations. Petit et al. (2001) reported increased first service conception rates of 87.5% in cows fed formaldehyde-treated flaxseed (17.0% of ration on a DM basis) as a fat source compared with 50.0% in cows fed a diet containing Megalac and flaxseed meal (5.6 and 12.7%, respectively). By using formaldehyde to protect the lipids in the whole flaxseed from biohydrogenation in the rumen, flaxseed was thought to increase conception rates by delivering a greater percentage of linolenic acid (an omega-3 fatty acid) to the animal thereby shifting fatty acid metabolism from the arachidonic acid pathway (utilized by omega-6 fatty acids) to the eicosapentaenoic acid pathway (utilized by omega-3 fatty acids) which is thought to inhibit prostaglandin series 2 production. Subsequent research (Petit et al., 2004) in dairy cattle examined the effects of feeding flaxseed on blood parameters and found that flaxseed fed dairy cattle had significantly lower omega-6:omega-3 fatty acid ratios than dairy cattle fed other sources of fat. As data showing the benefits of feeding flaxseed or LSM to livestock becomes more abundant, this feed product may become a more popular ingredient in a variety of types of feed. This makes it all the more important to fully understand the health implications of feeding flaxseed and LSM to livestock as well as their consumption in human diets. In addition to omega-3 fatty acids, the phytoestrogen SDG is also found in flaxseed and has exhibited many physiological effects on organisms consuming the product. Thus, SDG may have important implications in livestock production as flax becomes more frequently used in animal diets; however, although the impact of omega-3 fatty acids on livestock production has been extensively researched, data illustrating the impact of SDG on livestock production remains limited.
Phytoestrogens

Phytoestrogens are plant-derived, non-steroidal compounds capable of inducing estrogenic responses. There are three classes of phytoestrogens; isoflavones, lignans, and coumestans (Duncan et al., 2003). These compounds are heterocyclic phenols that resemble endogenous estrogens (Murkies et al., 1998). As a precursor to the mammalian lignans enterodiol and enterolactone, dietary SDG has been termed a phytoestrogen because of its potentially estrogenic and antiestrogenic properties. Consumption of SDG results in its metabolism to enterodiol and enterolactone by intestinal microflora (Axelson and Setchell, 1981) and possibly gastrointestinal enzymes such as lactase phlorizin hydrolase (Chen et al., 2005). The SDG is metabolized to enterodiol and enterolactone either directly, or through the intermediate matairesinol (Figure 1).

![Chemical structures](image)

Figure 1. Metabolism of SDG to enterodiol and enterolactone by intestinal microflora or endogenous enzymes.
Thompson et al. (1997) found SDG levels in flaxseed to be highly variable across year, location, and variety of flaxseed; however, flaxseed has been found to yield more enterodiol and enterolactone than any other oilseed or cereal grain investigated (Thompson et al., 1997). Enterodiol and enterolactone production range from 2.0 to 4.5 µmol/g with in vitro assays of flaxseed meal having yielded as much as 32 µmol/g for enterodiol and 68,204 µmol/g for enterolactone (Thompson et al., 1991).

**Physiological Effects Associated with Exposure to Phytoestrogens**

Effects of phytoestrogen consumption include reproductive, growth, developmental, anticarcinogenic, antioxidant, angiogenic, and anti-inflammatory effects among others. Some of these responses have been observed for SDG consumption whereas still others have been observed following consumption of other phytoestrogens. Their similarities to SDG is speculative as different phytoestrogens appear to have different effects on specific tissues. Adams (1995) reviewed a number of reports of phytoestrogen’s (this review focused on isoflavones and coumestans) effects on sheep and cattle. In both species, infertility, low calving and lambing rates, prolapse, irregular estrous cycles, and cystic ovaries were observed as a result of grazing of phytoestrogen-containing forages. Although the impact of phytoestrogens on livestock productivity is obvious, it is important to note that the effects of phytoestrogen consumption on livestock varies greatly depending on what class of phytoestrogen is being examined and the rate at which the compounds are being consumed. To my knowledge, there are no reports of decreased fertility due to consumption of lignans in livestock.
Altered Estrous/Menstrual Cycle Length

In a number of studies, females consuming phytoestrogens have altered estrous or menstrual cycles. Women consuming defatted flaxseed experienced a lengthened luteal phase, however overall menstrual cycle lengths were not altered (Phipps et al., 1993). This study also noted an increased progesterone:estrogen ratio and concluded that supplementation with flaxseed altered steroidogenic activity of the ovaries. Tou et al. (1998) reported that female rat pups born to dams fed a diet containing 10% flaxseed exhibited a lengthened estrous cycle due to a longer estrus phase. This persisted through d 132 where 20% of the females observed became anestrus due to persistent estrus. In contrast, about 14% of females born to dams fed a phytoestrogen-free diet in combination with a daily gavage of approximately 1.5 mg of SDG per day and about 17% of females from dams fed 5% flaxseed were anestrous due to persistent diestrus. This was compared with offspring from dams fed the basal diet which were all cycling at d 132.

Phipps et al. (1993) found that in healthy women with regular menstrual cycles, ingestion of a flaxseed supplement caused an increase in the length of the luteal phase of the menstrual cycle but did not significantly alter the lengths of the follicular phase or the overall menstrual cycle. Furthermore, this increase in luteal phase length is accompanied by a higher progesterone:estradiol-17β (E₂) ratio than women not supplemented with flaxseed due to a decrease in E₂ concentration during the luteal phase.

Alterations in Organ Mass

The uterotropic properties of E₂ have been well established for nearly 80 years (Loose and Stancel, 2006). The uterotropic effects of phytoestrogens have also been reported (Adams, 1995). Genistein has been reported to increase wet and dry weights of the
uterus and cervix (Ford et al., 2006). Tou et al. (1998) noted an increase in pregnant rat dam uterine and ovarian mass when rats were fed a diet containing 10% flaxseed. This effect carried over into the dam’s female offspring as well. Female offspring had heavier uteri at d 21 when dams were treated with purified SDG (given as a 1.5 mg/d gavage) or fed the 5% or 10% flaxseed diet. However, ovarian weights decreased when rats were fed the 5% flaxseed diet or treated with SDG. At d 50 and d 132, uterine weights were similar in all treatment groups whereas ovarian weights were increased when dams were fed the 10% flaxseed diet. Effects on reproductive organs were also documented in male offspring. At d 132, male offspring’s accessory sex glands and prostate glands were heavier when dams were fed 10% flaxseed. Also, histological examination of the prostate gland revealed an increase in cellular proliferation, secretory epithelial cells, papillary infoldings, and secondary projections in the ventral lobe of the prostate when dams were fed the 10% flaxseed diet. In contrast, male offspring from dams fed 5% flaxseed or gavaged with SDG exhibited minor declines in proliferation of the prostate.

Phytoestrogens have also been observed to alter the growth of non-reproductive organs. Genistein, the isoflavone found in soy products, inhibits cellular proliferation at high concentration and promotes cellular proliferation at low concentration. Investigators reported that piglets fed milk replacer supplemented with a high concentration (14 mg/L) of genistein exhibited a cellular proliferation rate within the crypt region of the jejunum that was nearly half that of piglets fed a milk replacer with no genistein added or a low concentration (1 mg/L) of genistein added (Chen et al., 2005).
**Fetal Development**

Offspring in utero are sensitive to many estrogenic compounds and protection of the fetus during sex-differentiation from estrogenic compounds is accomplished by binding-proteins such as α-fetoprotein and sex hormone binding globulin (SHBG; Tou et al., 1998). Estrogenic compounds such as diethylstilbestrol are detrimental to the fetus (Block et al., 2000; Kaufman et al., 2000; Palmer et al., 2001). Reports indicate that women exposed to diethylstilbestrol in utero were more likely to develop breast cancer (Calle et al., 1996); experience spontaneous abortions, preterm delivery, and ectopic pregnancy (Kaufman et al., 2000); suffer from infertility (Palmer et al., 2001); and be born with developmental abnormalities of the reproductive tract (Block et al., 2000). In addition to reproductive function, Migliaccio et al. (1996) observed diethylstilbestrol exposure in utero to affect bone mass, femur length, and bone calcium content in female mice. Males exposed to estrogenic compounds, including diethylstilbestrol, in utero often exhibit feminization, hypoplastic testes, and cryptorchidism (McLachlan, 2001) whereas Wilcox et al. (1995) observed as much as a three times greater likelihood to develop genital malformations as adults than men not exposed to diethylstilbestrol. The devastating effects of prenatal exposure to estrogenic compounds necessitate the evaluation of these compounds to ensure that their use does not lead to these consequences. In addition to fetal development in utero, evidence exists that exposure of male mice to estrogenic compounds such as diethylstilbestrol not only during gestation, but also during lactation can lead to a decrease in testicular function and fertility (Fielden et al., 2002).

With the abundance of data illustrating the sensitivity of the developing embryo to overexposure to environmental estrogens and the possible detrimental outcomes for both
the male and female offspring of exposing gestating females to environmental estrogens, it becomes apparent that the consumption of specific phytoestrogens should be thoroughly researched to identify the consequences associated with the particular estrogenic compound. There exists the possibility that the presence of lignans could negatively impact the outcome of pregnancy; however, the effects of SDG on offspring in utero remain both limited and conflicting. Tou et al. (1998) fed rat dams a phytoestrogen-free diet, a 5% and 10% flaxseed diet, and a phytoestrogen-free diet in conjunction with a daily gavage of SDG approximately equal to what the 5% flaxseed diet (1.5 mg/d) would likely contain. Neither flaxseed nor SDG ingestion at any level tested during pregnancy altered gestation length, litter size, percentage of pups born alive, or percentage of pups surviving to 21 d of age. However, they did observe that offspring born to dams fed the 10% flaxseed diet had lighter birth weights compared with offspring from dams fed the basal and 5% diets as well as those treated with purified SDG. Although this decreased birth weight was not observed in females after 3 d, it did persist in male offspring who experienced a smaller weight gain from d 3 until d 21. In contrast, female offspring had similar weights by d 3 and experienced similar weight gain in all treatment groups. Also, both male and female rat pups exposed to the 10% flaxseed diet in utero had shorter anogenital distances than rat pups exposed to both the basal and 5% flaxseed diets as well as purified SDG.

In addition to feeding flaxseed or purified SDG to pregnant rat dams during pregnancy, Tou et al. (1998) also investigated the potential transfer of SDG to rat pups through suckling. Twenty days following parturition, rat dams were gavaged with radio-labeled SDG and offspring were allowed to suckle for 24 h before whole body radioactivity of dams and pups were determined. Whereas whole body radioactivity was increased in rat
pups suckling dams gavaged with radio-labeled SDG indicating that there was transfer of radioactivity from the dam to the suckling pup, they did not determine if this radioactivity is due to radio-labeled SDG, enterodiol, enterolactone, or some other metabolite of SDG.

To determine the effects of feeding SDG both during pregnancy and during suckling, rat dams were fed the flaxseed and basal diets or treated with purified SDG throughout pregnancy as well as during lactation through d 21. They found that females born to dams fed the 10% flaxseed diet reached puberty at a younger age and lighter weight than females born to dams fed a basal diet whereas females born to dams fed the 5% diet or gavaged with SDG reached puberty at an older age and heavier weight than females born to dams fed the basal diet. In contrast Ward et al. (2001) found no effect of rat dams consuming LSM or purified SDG.

Angiogenesis

The development of new blood vessels through angiogenesis is a common biological process that occurs in developing tissues, healing wounds, placenta, and, in adult females, cyclically in reproductive organs (Fotsis et al., 1993, Reynolds and Redmer, 1998). Estrogen plays an integral role in regulating many cardiovascular processes. Estrogen both alters protein expression of molecules via genomic receptors that regulate angiogenesis and also induces a rapid vasodilatory response via non-genomic receptors of both arteries and veins (Mendelsohn, 2000; Bracamonte et al., 2002). Magness et al. (1998) reported that vascular resistance of various tissues including skin, kidney, brain, skeletal muscle, small bowel, and uterus in ovariectomized sheep injected with E2 decreased concomitantly with an increase in blood flow. In cattle, the sudden increase and subsequent decline in E2 levels observed during estrus alter blood flow to the endometrium to the
extent that caruncular capillaries hemorrhage (Youngquist, 2007). The dramatic increase in uterine blood flow observed during pregnancy has also been linked to increased levels of circulating E\textsubscript{2} associated with pregnancy (Magness et al., 2005). In addition to angiogenesis in normal tissue, angiogenesis is also necessary to the pathologic development of tumor cells (Liu and Deisseroth, 2006).

Indeed, the use of the antiestrogenic compounds has been reported to inhibit angiogenesis. The use of the antiestrogenic cancer drug tamoxifen inhibits tumor growth at least in part by inhibiting angiogenesis in rat models (Blackwell et al., 2000) and in vitro human breast cancer models (Haran et al., 1994; Chen et al., 2004). Phytoestrogens also play a role in inhibiting angiogenesis. Kruse et al. (1997) reported that the phytoestrogen genistein injected subconjunctivally inhibited basic fibroblast growth factor induced neovascularization of the limbus of the eye in rabbits. Furthermore, in vitro studies indicate that genistein may inhibit angiogenesis in tumors (Fotsis et al., 1993). Phytoestrogens’ ability to alter angiogenesis also offers another explanation to SDG’s ability to alter the length of estrous and menstrual cycles (Phipps et al., 1993); the ability of phytoestrogens to alter angiogenesis results in altered blood flow to the corpus luteum, follicle, or ovary as a whole. Angiogenesis is key to controlling the development and atresia of structures on the ovary (Dubey et al., 2000) and vascularization of the corpus luteum is necessary for proper corpus luteum function and progesterone production (Redmer and Reynolds, 1996).

**Phytoestrogens and the Digestive Tract**

Although estrogens are often associated with the reproductive system, these compounds may affect non-reproductive organ systems as well. Not only have estrogen receptors have been observed in the digestive tract (Thomas et al., 1993) but there are
indications that hormone status may alter vascular dynamics and structural characteristics of the gastrointestinal tract. As previously mentioned, Magness et al. (1998) noted an increase in blood flow to the small intestine in ovariectomized ewes subjected to prolonged exposure to E2. Additionally, Cripps and Williams (1975) observed an increase in small intestinal wet weight and duodenal villus length in pregnant rats.

Not only is the digestive tract necessary for life, but altering its characteristics can induce changes in the growth and health of organisms. Additionally, altering the function of the digestive tract early in life or during fetal development may lead to changes in structure or function that persist throughout the life of the individual. In the absence of phytoestrogen induced alterations of the fetal digestive tract, alterations in the digestive tract of the dam that lead to a change in nutritional status may also impact the fetus. It has been shown that altering maternal nutrient intake during pregnancy can have a lifelong negative impact on the fetus (McMillen and Robinson, 2005). For these reasons, it is important to consider the possible impact that feeding phytoestrogens may have on the structure and function of the digestive tract. If these effects are being mediated through E2, either directly or indirectly, there exists the possibility that an E2 agonist or antagonist could interfere with the normal physiology of these tissues. As previously discussed, this has been demonstrated in piglets fed the phytoestrogen genistein (Chen et al., 2005) in which cellular proliferation was decreased in piglets fed a genistein containing diet.

**Mechanisms of Phytoestrogen's Effects**

The tissue response elicited by phytoestrogen exposure is quite variable. Animal species as well as the type and dose of phytoestrogen being consumed may alter the observed tissue response to phytoestrogens. Furthermore, the response observed in an
animal may also depend upon the tissue being investigated. For this reason, theorizing the mechanism by which these compounds produce their effects becomes difficult at best. Phytoestrogens are thought to mediate their physiological effect via a number of mechanisms. Phytoestrogens may interact with $E_2$ receptors as agonists of $E_2$ or compete with $E_2$ for receptor binding thus inhibiting $E_2$’s effects. There are also data indicating that phytoestrogens increase plasma SHBG concentration which then sequesters a greater proportion of plasma $E_2$ making less $E_2$ available for $E_2$ receptor binding. Phytoestrogens may also alter the production, degradation, and clearance of endogenous steroid hormones (Adlercreutz et al., 1987; Whitten and Shultz, 1988; Martin et al., 1996; Rosselli et al., 2000).

Phytoestrogens can be classified as selective estrogen response modulators, commonly abbreviated SERMs. These compounds interact with $E_2$ receptors and can act selectively as $E_2$ agonists or antagonists depending on what tissue is being investigated (Loose and Stancel, 2006). Indeed, Collins et al. (1997) demonstrated in vitro that estrogenic and antiestrogenic activity mediated via the $E_2$ receptor varied depending upon the phytoestrogen being examined and also that these compounds bound to the $E_2$ receptor with varied affinities. Collins et al. (1997) examined a number of phytoestrogens’ (they did not include SDG) ability to interact with the human estrogen receptor in vivo. They found that the phytoestrogens tested were able to interact with the human estrogen receptor and displace the binding of $E_2$ at varying concentrations. They also found that some phytoestrogens were able to induce transcription of an $E_2$ reporter gene, but to a lesser extent than $E_2$ itself. In addition, they found that some of the phytoestrogens were able to inhibit $E_2$ induced transcription of a reporter gene. These results clearly show that
phytoestrogens can act either as an agonist, antagonist, or both depending upon the specific phytoestrogen being examined, the presence or absence of E$_2$, the concentration of phytoestrogen being used, and the biological system or tissue being examined.

Reports of phytoestrogen’s ability to alter SHBG concentration are variable. Martin et al. (1996) found that a number of phytoestrogens (SDG was not among them) were able to bind SHBG and proposed that not only do phytoestrogens compete with E$_2$ for E$_2$ receptor binding sites, but phytoestrogens may also compete for SHBG binding sites thereby making more E$_2$ available for E$_2$ receptor binding. On the other hand, the hypothesis also opens the possibility that with more free E$_2$ circulating in the plasma, more E$_2$ would be available for metabolism resulting in an increased clearance rate. In vitro studies (Loukovaara et al., 1995) report that the isoflavonoids daidzein, equol, and genistein increased intracellular concentrations of SHBG, however genistein failed to increase extracellular levels of SHBG as daidzein and equol did.

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effects of environmental oestrogens: Implications for reproductive and vascular


**Statement of the Problem**

The properties of phytoestrogens have been researched extensively. Their use has been postulated in a wide range of applications such as an anticarcinogenic compound, having a role in hormone replacement therapy, and improving fertility and growth in livestock. Currently, much of the data available focuses on soy products and isoflavones, rather than flax and SDG. Additionally, much of the flax research has been conducted using whole flax or flax meal and has focused on effects of linseed oil rather than the lignan component of flaxseed, although rodent models have been used to some extent to investigate the estrogenic properties of LSM and purified SDG. The investigation of linseed meal on the biology of livestock is necessary to further explore the application of LSM as a feed source in the livestock industry.

Due to the estrogenic nature of LSM, and the cyclic nature of E₂ production in the intact cyclic female, the ovariectomized ewe will be used as a model to eliminate the possibility of endogenous E₂ confounding collected data. Additionally, E₂ containing
Silastic implants will be used to investigate possible interactions of LSM with E\textsubscript{2} as well as any antiestrogenic properties of LSM. Although this model cannot substitute models using intact cycling females, it can provide insight to LSM’s effects on the adult ewe, its interactions with E\textsubscript{2}, and possible mechanisms of action which can then be used in further applied studies of the intact animal.
CHAPTER 2

ESTRADIOL-17β AND LINSEED MEAL INTERACT TO ALTER VISCERAL ORGAN MASS IN OVARIECTOMIZED EWES


Abstract

To evaluate the estrogenic potential of SDG found in LSM on visceral organ and uterine mass, 48 multiparous, ovariectomized ewes (54.6 ± 1.1 kg) were used in a 3 x 4 factorial. Main effects were length of LSM feeding (0, 1, 7, or 14 d) and length of exposure to E₂ implant (0, 6, or 24 h before tissue collection). Implanting ewes with E₂ for 24 h increased liver mass relative to empty body weight (EBW; g/kg EBW) compared with ewes implanted for 0 or 6 h (P ≤ 0.03); whereas feeding LSM for 14 d decreased liver mass compared with ewes fed LSM for 1 or 7 d (P ≤ 0.02). There was a LSM x E₂ interaction (P = 0.02) for uterine mass (g/kg BW). Ewes implanted with E₂ for 24 h had increased uterine mass compared with ewes implanted for 0 h when LSM was fed for 0, 7, or 14 d (P ≤ 0.01), uterine mass increased (P = 0.02) when ewes were implanted for only 6 h when LSM was fed for 1 d. There was a LSM x E₂ interaction (P = 0.01) for duodenal mass (g/kg EBW). When fed LSM for 0 d, implanting ewes with E₂ for 24 h increased (P = 0.02) duodenal mass compared with ewes implanted for 0 h. Feeding LSM for 14 d decreased duodenal mass when ewes were implanted for 6 or 24 h compared with ewes implanted for 0 h (P ≤ 0.04). When ewes were implanted with E₂ for 0 h, feeding LSM for 1, 7, or 14 d increased (P ≤ 0.02) duodenal mass compared with ewes fed LSM
for 0 d and when LSM was fed for 0 d, implanting ewes with E₂ for 24 h increased \( (P = 0.02) \) duodenal mass compared with ewes implanted for 0 h. Stomach complex mass (kg) also tended \( (P = 0.08) \) to increase when LSM was fed for 7 d but when fed for 14 d, mass was similar \( (P = 0.47) \) to that observed when LSM fed for 0 d. Neither feeding LSM nor implanting with E₂ altered the mass (g/kg EBW) of jejunum \( (P \geq 0.36) \), ileum \( (P \geq 0.28) \) or percentage jejunal mucosa \( (P \geq 0.15) \). Additional research investigating the effects of feeding LSM on intact cyclic ewes and the further investigation of the mechanisms through which LSM elicits its effects is warranted.

**Introduction**

The inclusion of flaxseed (*Linum unisatissimum*) in diets of pregnant rats decreased birth weight and variably altered reproductive organ weight, anogenital distance, and age and weight at puberty of rat pups depending on dosage and sex of offspring (Tou et al., 1998). The addition of flaxseed to dairy cattle diets (Petit et al., 2001; Petit et al., 2004) increased conception rates and including flaxseed in feedlot diets increased ADG and feed efficiency, and improved carcass quality grades (Maddock et al., 2006). The exact mechanisms by which flaxseed can affect growth and reproductive performance is not known. Whereas many of these characteristics are thought to be attributable to the large concentration of omega-3 fatty acids found in flaxseed, the mammalian lignan precursor SDG may also have a role. Lignans, including SDG, are a member of a class of compounds termed phytoestrogens due to their ability to elicit cellular responses via estrogen receptors (Collins et al., 1997). Although it has not been shown to interact with the estrogen receptor directly, SDG is a precursor to enterodiol and enterolactone (Axelson et al., 1982; Rickard et al., 1996; Begum et al., 2004). When SDG is consumed, intestinal microflora (Axelson...
and Setchell, 1981) and gastrointestinal enzymes, such as lactase phlorizin hydrolase (Day et al., 2000), break down SDG to enterodiol and enterolactone which are then absorbed in the intestine and able to interact with the estrogen receptor systemically. Due to the proposed estrogenic effects of SDG, feeding LSM may alter visceral organ mass such as the liver (Rumsey et al., 1996; Hutheson et al., 1997) or uterus (Reynolds et al., 1998) as well as circulating concentrations of hormones responsible for growth such as insulin-like growth factor-I (IGF-I; Johnson et al., 1996).

The objectives of this study were to determine how the length of LSM feeding and E₂ exposure affect visceral organ mass, including the liver, small intestine, stomach and uterus as well as circulating concentrations IGF-I, triiodothyronine (T₃), and thyroxine (T₄) in ovariectomized ewes.

**Materials and Methods**

**Animals and Treatments**

All experimental protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee before the initiation of the research. Ewes were housed indoors at the North Dakota State University Animal Nutrition and Physiology Center for the duration of the experiment (early December through mid January). Light-dark cycles were timer-controlled and were set to coincide with sunrise and sunset at this location. Forty-eight multiparous ewes (54.6 ± 1.1 kg initial BW) of mixed breeding were fed in groups of 9 to 11 in pens measuring 3.04 × 3.73 m, ovariectomized via midventral laparotomy (Reynolds et al., 1998), and allowed to recover for at least 28 d before treatments were initiated. After ovariectomy, ewes were fed an LSM free diet (Control diet; Table 2) until initiation of treatment. The feeding of a LSM free diet
following ovariectomy was carried out to ensure that any circulating endogenous estrogens as well as any dietary SDG was cleared from the body before treatments were initiated.

Ewes were fed to meet their requirement for maintenance for each pen based on their metabolic body weight (NRC, 1985). Ewes were weighed every two weeks, had ad libitum access to water, and were fed once daily at 0800 throughout the experiment.

Table 2. Diet formulation and analyzed dietary composition

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>LSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% as-fed basis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beet pulp</td>
<td>81.25</td>
<td>87.50</td>
</tr>
<tr>
<td>Dried distiller's grain</td>
<td>17.00</td>
<td></td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>Linseed meal</td>
<td></td>
<td>12.50</td>
</tr>
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</table>

Analyzed dietary nutrient content

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>LSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>89.22</td>
<td>90.51</td>
</tr>
<tr>
<td>OM</td>
<td>93.65</td>
<td>93.24</td>
</tr>
<tr>
<td>CP</td>
<td>11.77</td>
<td>12.31</td>
</tr>
<tr>
<td>NDF</td>
<td>43.18</td>
<td>41.34</td>
</tr>
<tr>
<td>ADF</td>
<td>24.13</td>
<td>24.78</td>
</tr>
<tr>
<td>Fat</td>
<td>2.27</td>
<td>1.22</td>
</tr>
<tr>
<td>IVDMD(^2)</td>
<td>85.86</td>
<td>86.91</td>
</tr>
<tr>
<td>IVOMD(^3)</td>
<td>84.76</td>
<td>86.11</td>
</tr>
<tr>
<td>Ca</td>
<td>0.64</td>
<td>0.92</td>
</tr>
<tr>
<td>P</td>
<td>0.23</td>
<td>0.24</td>
</tr>
</tbody>
</table>

\(^1\) Linseed meal.
\(^2\) In vitro dry matter disappearance.
\(^3\) In vitro organic matter disappearance.

Immediately before initiation of treatment, ewes were moved to individual pens measuring 1.52 x 1.87 m. Ewes were weighed and stratified by BW into blocks (i.e., the 12 lightest ewes were assigned to block 1, the next 12 lightest ewes were assigned to block 2, etc.). Once stratified, ewes within a block were assigned randomly to each treatment group.
Treatments were arranged as a 3 x 4 factorial. Main effects consisted of length of feeding a diet containing 12.5% LSM (as-fed basis; Table 2) and length of exposure to E₂ via subcutaneous implant. The LSM diet was designed to be SDG free, with the exception of the inclusion of 12.5% LSM. Laboratory analysis of the LSM used to formulate the LSM diet indicated the SDG content of the LSM diet to be 14.53 mg SDG per g of feed on a DM basis. Control and LSM diets were formulated to be similar in calculated protein (13.7 and 13.6% CP, respectively) and net energy (1.80 and 1.79 Mcal/kg of diet, respectively). Both diets were pelleted and ewes were limit fed to meet the net energy requirements for maintenance based on ewe metabolic BW (56 kcal/kg^0.75; NRC, 1985). Ewes were either fed the Control diet throughout the experiment until tissues were collected or, after being fed the Control diet diet for at least 1 month, were fed the LSM diet for 1, 7, or 14 d before tissue collection. Ewes within each duration of LSM feeding were implanted with E₂ for 0, 6, or 24 h before tissue collection. Thus, each treatment group consisted of 4 ewes. Ewes receiving E₂ implants were locally anesthetized and implanted subcutaneously in the axillary region as validated by Johnson et al. (1997a). Implanted ewes each received two Silastic implants (3.35 mm i.d. x 4.65 mm o.d. x 15 mm length; Dow Corning, Midland, MI) each containing 50 mg of E₂ (Sigma-Aldrich, St. Louis, MO). After implantation, the incision was closed with surgical staples and a topical antibiotic was applied to the incision site. The implants remained until tissue collection, at which time their presence was confirmed.

**Blood and Tissue Collection**

Serum and plasma samples were collected from ewes fed LSM via jugular venipuncture 1 d before initiation of LSM feeding. Serum and plasma was also collected
from E2-implanted ewes immediately before being implanted with E2 and from all ewes 1 h before tissue collection. Serum tubes were allowed to clot on ice for approximately 2 to 3 h after which both serum and plasma tubes were centrifuged at 1,500 × g for 20 min at 4°C. Serum and plasma were then pipetted into glass tubes and stored at -20°C until further analysis.

At the time of tissue collection, ewes were stunned via captive bolt and exsanguinated. The reproductive tract was removed and the oviducts, mesometrium, and cervix were removed from the uterus and uterine weight was recorded. A sample of caruncular tissue was collected from the uterus, snap-frozen in liquid nitrogen, and stored at -70°C for later DNA, RNA, and protein analysis. The viscera were removed from the body cavity, the gallbladder was removed from the liver, and the liver was weighed. A sample of the liver (approximately 50 to 75 g) from the left liver lobe was snap-frozen in liquid nitrogen and stored at -70°C for further analysis.

Procedures to locate demarcations of the small intestine were modified from those of Scheaffer et al. (2004). Briefly, the duodenum was designated as the region of the small intestine extending from the pyloric valve to a point directly adjacent to the branch of the gastroplenic and anterior mesenteric veins. The jejunum was demarcated as the region of the small intestine extending from the caudal end of the duodenum to a point determined by measuring 15 cm distally along the mesenteric vein from the branch of the ileocecal and mesenteric veins and following the vascular arcade to an adjacent point on the small intestine. From this point, an additional 300 cm was measured caudally to demarcate the caudal end of the jejunum. In addition, approximately 10 cm of jejunal tissue was subsampled from the cranial end of the distal 300 cm of jejunum for analysis of percentage
mucosa and mucosal DNA, RNA, and protein. The remaining section of the small intestine extending from the caudal end of the jejunum to the cecum was considered the ileum. All components of the small intestine were removed from the mesentery, cleaned of digesta, and weighed. After the removal of the omentum and digesta, the stomach complex was weighed. Digesta from the small intestine and stomach complex was combined with digesta removed from the cecum and large intestine and weighed.

**Tissue Analysis**

Samples of liver, uterine, and jejunal tissues were weighed and lyophilized (Genesis 25 LE; The Virtis Co., Gardiner, NY) to determine dry weight. Liver, jejunal mucosa, and caruncular tissues were analyzed for DNA, RNA, and protein content, as previously reported (Bradford, 1976; Johnson et al., 1997b). A single caruncular tissue sample was used as a control in the DNA assay, an assay standard solution (12.5 mg of RNA/mL) was used as a control in the RNA assay, and the Accutrol normal control (Sigma-Aldrich, St. Louis, MO) was used as a control in the protein assay. Intra- and interassay CV were 4.87 and 5.97%; 7.08 and 0.69%; 5.11 and 1.13% for DNA, RNA, and protein assays, respectively. The RNA:DNA ratio was used as an index of cellular activity whereas the protein:DNA ratio was used as an index of hypertrophy.

**Hormone Analysis**

Benzene was used to extract E₂ from 1 mL of plasma, and E₂ concentrations were determined by RIA as previously reported (Redmer et al., 1991). Samples were analyzed across two assays and the intra- and interassay CV were 2.14 and 2.00%, respectively. The lower limit of detection for the RIA was 1.2 pg/mL.
Plasma IGF-I and serum T₃ and T₄ levels were determined using an Immulite 1000 (Siemens, Los Angeles, CA). Plasma IGF-I concentrations were analyzed using a commercial kit (Cat # LKGFS; Siemens, Los Angeles, CA) as directed by the manufacturer. Plasma samples were prepared in a 1:10 dilution with the IGF-I pretreatment solution and analyzed in duplicate in a single assay. Intra-assay CV was 4.9%. Serum T₃ and T₄ concentrations were also determined using commercial kits (Cat # LKT41 and LKT31, respectively; Siemens, Los Angeles, CA) as instructed by the manufacturer. Assays for T₃ and T₄ were run in duplicate in two assays. Intra- and interassay CV were 6.9 and 4.8% and 7.8 and 5.8% for T₃ and T₄, respectively.

**Statistics and Calculations**

Data for ewe BW, organ mass, cellular measurements, and hormone concentrations were analyzed as a 3 × 4 factorial in a randomized complete block design using PROC MIXED (SAS Inst. Inc., Cary, NC). The model statement included effects of block (based on initial BW), length of LSM feeding, length of E₂ exposure, and the interaction of LSM feeding and E₂ exposure. Fixed variables were length of LSM feeding, length of E₂ exposure, and block. The diagonal covariate structure was used. Organ masses are presented as organ mass and organ mass per unit of EBW (ewe live BW at slaughter minus digesta weight). To calculate organ mass as a percentage change from 0 h E₂, the difference in organ mass of each ewe from the mean organ mass of the respective 0 h E₂ groups within each duration of LSM was divided by the mean organ mass of the respective 0 h E₂ group. Hormone data were analyzed as a repeated measure where appropriate with ewe nested within treatment as the subject. Initial blood sampling hormone concentration was used as a covariate when it explained a significant (P ≤ 0.05) portion of the variation. Data
are presented as least squares means ± SEM. Mean separations were performed via LSD which were protected by an overall treatment $F$-test at $P = 0.05$. Differences were considered significant if $P \leq 0.05$, unless otherwise stated.

**Results**

Over the course of the study two ewes were removed for health reasons, one from the treatment that was fed LSM for 0 d and implanted with E$_2$ for 0 h and one from the treatment that was fed LSM for 7 d and implanted with E$_2$ for 0 h. The health concerns of these ewes were unrelated to the treatments imposed in the current study, the treatments, or any functions of the treatments. In addition to the aforementioned ewes, liver mass was mis-recorded for one ewe from the treatment group fed LSM for 0 d and implanted with E$_2$ for 6 h, therefore liver mass data for that ewe was not used in the statistical analysis.

Initial ewe BW taken at time of stratification averaged 54.6 ± 1.1 kg and did not differ among ewes assigned to each length of LSM ($P = 0.45$) feeding or each length of time implanted with E$_2$ ($P = 0.87$; Table 2). At slaughter, ewe BW averaged 51.6 ± 1.0 kg and did not differ due to length of LSM feeding ($P = 0.45$) or length of time implanted with E$_2$ ($P = 0.37$).

**Estrogen Concentration**

Plasma E$_2$ concentrations were at or below the detection limit before LSM feeding and at time of slaughter in ewes implanted with E$_2$ for 0 h as well as before E$_2$ implant in ewes that were implanted for 6 or 24 h (data not shown). Ewes implanted with E$_2$ for 6 or 24 h experienced an increase ($P < 0.001$; 81.1 and 61.4 ± 5.1 pg/mL respectively) in plasma E$_2$ concentrations at slaughter compared with ewes implanted with E$_2$ for 0 h.
Implanting ewes with E$_2$ tended to increase ($P = 0.10$) liver mass (g) whereas feeding LSM decreased ($P = 0.03$) liver mass (Table 3). Liver mass of ewes fed LSM for 14 d ($613.7 \pm 18.2$ g) was decreased compared with liver mass of ewes fed LSM for 1 ($P = 0.04; 668.8 \pm 18.2$ g) or 7 d ($P = 0.004; 695.4 \pm 19.3$ g) and was similar to liver mass of ewes fed LSM for 0 d ($P = 0.22; 647.5 \pm 20.3$ g). Expressing liver mass as organ mass per EBW (g/kg EBW) yielded similar responses to feeding LSM ($P = 0.03$) and implanting with E$_2$ ($P = 0.01$). Although liver mass of ewes fed LSM for 7 d ($14.23 \pm 0.33$ g/kg EBW) was similar to liver mass of ewes fed LSM for 0 ($P = 0.41; 13.84 \pm 0.34$ g/kg EBW) or 1 d ($P = 0.70; 14.06 \pm 0.31$ g/kg EBW), feeding LSM for 14 d ($12.93 \pm 0.31$ g/kg EBW) tended ($P = 0.06$) to decrease liver mass compared with ewes fed LSM for 0 d and decreased liver mass compared with ewes fed LSM for 1 or 7 d ($P = 0.02$ and $P = 0.01$, respectively). Contrary to the effects of LSM, implanting ewes with E$_2$ for 24 h increased liver mass ($14.48 \pm 0.27$ g/kg EBW) compared with ewes implanted for 0 ($P = 0.004; 13.22 \pm 0.29$ g/kg EBW) or 6 h ($P = 0.03; 13.59 \pm 0.28$ g/kg EBW) whereas implanting ewes for 6 h did not alter ($P = 0.37$) liver mass compared with ewes implanted for 0 h. Feeding LSM tended ($P = 0.06$) to alter the percentage DM of the liver. The percentage DM was reduced ($P = 0.02$) in ewes fed LSM for 7 d ($30.8 \pm 0.4\%$) compared with ewes fed LSM for 0 d ($32.2 \pm 0.4\%$). When LSM was fed for 14 d, the percentage DM of the liver was similar ($P = 0.73; 32.0 \pm 0.4\%$) to that observed when LSM fed for 0 d.

Percentage change in liver mass (g/kg EBW) of ewes implanted with E$_2$ for 6 and 24 h compared with ewes implanted with E$_2$ for 0 h was altered both through feeding LSM ($P = 0.002$) and implanting ewes with E$_2$ ($P = 0.05$; Figure 2). Implanting ewes that were
Table 3: Effect of LSM and E₂ on liver, uterine, and visceral organ mass

<table>
<thead>
<tr>
<th>Item</th>
<th>Days fed LSM</th>
<th>0 d</th>
<th>1 d</th>
<th>7 d</th>
<th>14 d</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item</td>
<td>Hours of E₂ exposure</td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
<td>0 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Ewe start wt. kg</td>
<td></td>
<td></td>
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<td>2.47</td>
<td>1.99</td>
<td>2.89</td>
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*Within a row, means with different superscripts differ (P ≤ 0.05).

1 Treatments were days of linseed meal feeding (LSM; 0, 1, 7, and 14 d) and length of estradiol-17β exposure (E₂; 0, 6, and 24 h).
2 Grams of tissue per kilogram of empty BW (Empty BW = BW minus blood and digesta Wt).
3 Stomach weight is the combined weight of the rumen, reticulum, omasum, and abomasum.
4 Means represent the percentage of the mass of the jejunum present in the mucosal scrape.
Figure 2. The percentage change in liver mass (g/kg EBW) of ewes implanted with E\(_2\) for 6 and 24 h compared with ewes implanted with E\(_2\) for 0 h, as determined by calculating the percentage difference of liver mass (g/kg EBW) of each ewe compared with that of ewes implanted with E\(_2\) for 0 h within respective durations of LSM feeding. Asterisks indicate that the percentage change was different ($P \leq 0.05$) from zero. Bracketed groups with different superscripts differ ($P < 0.05$).

Fed LSM for 0 or 1 d with E\(_2\) for 24 h increased ($P = 0.003$) liver mass (g/kg EBW) as a percentage of ewes exposed to E\(_2\) for 0 h, whereas 6 h of E\(_2\) exposure did not differ ($P \geq 0.11$) from the 0 h. However, when LSM was fed for 7 or 14 d, the resultant percentage change in liver mass due to implanting with E\(_2\) for 6 or 24 h decreased ($P \leq 0.03$) compared with ewes fed LSM for 0 or 1 d.

There was a LSM by with E\(_2\) interaction ($P \leq 0.02$) in uterine mass (g and g/kg; Table 3). When LSM was fed for 0 d, implanting ewes with E\(_2\) for 24 h increased uterine mass compared with ewes implanted with E\(_2\) for 0 h or 6 h. In ewes fed LSM for 1 d, uterine mass increased for ewes exposed to E\(_2\) for 6 h and further increased with 24 h E\(_2\) exposure. Uterine mass obtained from ewes fed LSM for 1 d and 24 h E\(_2\) exposure were heavier than uterine mass obtained from ewes in all other treatment combinations.
Uterine responses to E₂ after 7 and 14 d of LSM feeding returned to that observed when LSM was fed for 0 d. Although feeding LSM for 7 or 14 d and implanting ewes with E₂ for 6 h did not alter uterine mass compared with ewes implanted with E₂ for 0 h, 24 h E₂ exposure increased ($P \leq 0.02$) uterine mass compared with ewes implanted with E₂ for 0 and 6 h. Unlike liver tissue, LSM did not alter uterine DM percentage whereas implanting ewes with E₂ for 6 or 24 h decreased ($P \leq 0.01$) uterine DM percentage compared with ewes implanted with E₂ for 0 h. A further decrease ($P < 0.001$) in the percentage DM of the uterus was observed in ewes implanted with E₂ for 24 h compared with ewes implanted with E₂ for 6 h.

Both length of E₂ implant and length of feeding LSM altered ($P < 0.001$) the percentage change in uterine mass (Figure 3). Implanting ewes with E₂ for 6 h did not alter ($P \geq 0.12$) uterine mass as a percentage of the 0 h E₂ group within the respective duration of LSM feeding when LSM was fed for 0, 7, or 14 d. whereas 1 d of LSM feeding and 6 h E₂ exposure increased ($P = 0.001$) uterine mass. Further, within each d of LSM feeding, implanting with E₂ for 24 h increased ($P \leq 0.009$) uterine mass as a percentage of uterine mass of ewes exposed to E₂ for 0 h. Implanting ewes with E₂ and feeding LSM for 1 d resulted in increased percentage change in uterine mass compared with 0, 7, or 14 d LSM feeding (Figure 3). Further, implanting with E₂ for 24 h increased the percentage change in uterine mass compared with ewes implanted for 6 h and fed LSM for 0, 1, or 14 d.

Feeding LSM tended ($P = 0.06$) to alter the mass (kg) of the stomach complex. Stomach complex mass was greater ($P = 0.01$) in ewes fed LSM for 7 d (1.52 ± 0.03 kg) than in ewes fed LSM for 14 d (1.40 ± 0.03 kg) and tended ($P = 0.08$) to be greater than
in ewes fed LSM for 0 d (1.43 ± 0.03 kg). Stomach complex mass of ewes fed LSM for 1 d (1.49 ± 0.03 kg) was similar (P = 0.53) to ewes fed LSM for 7 d. Ewes fed LSM for 14 d had similar (P = 0.47) stomach complex mass as those fed LSM for 0 d. However, when stomach complex mass was scaled to EBW (g/kg EBW), there was no effect of LSM (P = 0.24).

Figure 3. The percentage change in uterine mass (g/kg EBW) of ewes implanted with E₂ for 6 and 24 h compared with ewes implanted with E₂ for 0 h, as determined by calculating the percentage difference of uterine mass (g/kg EBW) of each ewe compared with that of ewes implanted with E₂ for 0 h within respective durations of LSM feeding. Asterisks indicate that the percentage change was different (P ≤ 0.05) from zero. Bracketed groups with different superscripts differ (P < 0.05). Daggers indicate that the means within a bracketed group differ (P < 0.05).

There were LSM × E₂ interactions for duodenal mass (P = 0.02 and 0.01, respectively for g and g/kg EBW). Implanting ewes with E₂ for 24 h increased duodenal mass (g and g/kg EBW; P ≤ 0.05) compared with ewes implanted with E₂ for 0 h when LSM was fed for 0 d. After 1 d of feeding LSM, implanting ewes with E₂ for 6 or 24 h did not alter (P ≥ 0.30) duodenal mass (g and g/kg BW) compared with ewes implanted
for 0 h. This was followed by a reversal of the effect of E2 when LSM was fed for 7 or 14 d from what was observed when LSM was fed for 0 d. Duodenal mass (g and g/kg EBW) decreased when ewes were implanted with E2 for 24 h compared with ewes implanted with E2 for 6 h when LSM was fed for 7 d ($P = 0.04$ and $P = 0.05$, respectively). This effect occurred more rapidly when LSM was fed for 14 d as duodenal mass (g and g/kg EBW) decreased ($P \leq 0.005$) after only 6 h exposure to E2 implant compared with ewes not implanted with E2. When E2 was implanted for 0 h, it is also evident that feeding LSM increased ($P < 0.02$) duodenal mass (g and g/kg EBW) when fed for 1 d compared with ewes fed LSM for 0 d. Continued feeding for 7 and 14 d did not have a further effect on duodenal mass.

There were no differences observed between 6 and 24 h E2 exposure within each d of LSM feeding for the percentage change in duodenal mass (Figure 4). Implanting ewes with E2 did not alter ($P = 0.42$) duodenal mass (g/kg EBW) as a percentage of the 0 h E2 duodenal mass when LSM was fed for 1 d. Percentage duodenal mass change in response to E2 implant for 6 or 24 h when LSM was fed for 1 d was similar ($P = 0.33$) to that observed when LSM was fed for 7 d but less ($P < 0.001$) than the percentage change observed when LSM was not fed ($P < 0.001$). Feeding LSM for 14 d further decreased ($P < 0.001$) percentage change in duodenal mass that was observed when LSM was fed for 1 d or 7 d ($P \leq 0.05$) in response to implanting ewes with E2 for 6 and 24 h.

After changes in mass observed in the stomach complex and duodenum in response to feeding LSM and implanting ewes with E2, no further changes in mass were observed in the small intestine. Neither feeding LSM nor implanting ewes with E2 altered
jejunal mass (g or g/kg; $P \geq 0.11$ and $P \geq 0.23$, respectively) or ileal mass (g or g/kg; $P \geq 0.29$ and $P \geq 28$, respectively). Also, the percentage of the jejunum that was mucosa was not altered either by feeding LSM or implanting ewes with E$_2$ ($P \geq 0.15$).

Figure 4. The percentage change in duodenal mass (g/kg EBW) of ewes implanted with E$_2$ for 6 and 24 h compared with ewes implanted with E$_2$ for 0 h, as determined by calculating the percentage difference of duodenal mass (g/kg EBW) of each ewe compared with that of ewes implanted with E$_2$ for 0 h within respective durations of LSM feeding. Asterisks indicate that the percentage change was different ($P \leq 0.05$) from zero. Bracketed groups with different superscripts differ ($P < 0.05$).

**DNA, RNA, and Protein**

In the liver, feeding LSM and/or implanting ewes with E$_2$ did not alter ($P \geq 0.09$) concentrations of DNA, RNA, or protein, or RNA:DNA or protein:DNA ratios (Table 4). Although LSM feeding did not impact cellularity measurements of the caruncle, caruncular DNA concentration was greater in ewes not implanted with E$_2$ (6.62 ± 0.46 mg/g) than in ewes implanted for 6 h ($P = 0.01$; 5.02 ± 0.42 mg/g) or 24 h ($P < 0.001$; 3.82 ± 0.42 mg/g). Ewes implanted for 6 h also had greater ($P = 0.05$) caruncular DNA concentration than ewes implanted for 24 h. Caruncular protein
Table 4. Effect of LSM and E$_2$ on RNA, DNA, and protein concentration (mg/g), RNA:DNA ratio, and protein:DNA ratio in the liver, caruncle, and jejunal mucosa scrape

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<th>7 d</th>
<th>14 d</th>
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<td>24 h</td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
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</tr>
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<td>0.77</td>
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Within a row, means with different superscripts differ (P ≤ 0.05).

1 Treatments were days of linseed meal feeding (LSM; 0, 1, 7, and 14 d) and length of estradiol-17β exposure (E$_2$; 0, 6, and 24 h).

2 Mucosa scrape from the jejunum.
remained unaffected due to LSM \((P = 0.47)\) and E\(_2\) \((P = 0.14)\). Although caruncular RNA:DNA ratios were similar \((P = 0.20)\) between ewes not implanted \((0.32 \pm 0.07)\) and those implanted for 6 h \((0.45 \pm 0.07)\), implanting ewes for 24 h \((0.56 \pm 0.07)\) increased \((P = 0.02)\) the RNA:DNA ratio compared with ewes not implanted with E\(_2\) but did not increase \((P = 0.21)\) the RNA:DNA ratio compared with ewes implanted for 6 h. A similar, but more pronounced, effect was observed in the caruncular protein:DNA ratio in response to E\(_2\) \((P < 0.001)\). Caruncular protein:DNA ratios tended \((P = 0.08)\) to be greater in ewes implanted with E\(_2\) for 6 h \((1.35 \pm 0.17)\) compared with ewes not implanted with E\(_2\) \((0.90 \pm 0.18)\). Caruncular protein:DNA ratios further increased \((P = 0.01)\) when ewes were implanted for 24 h \((2.00 \pm 0.17)\) compared with ewes implanted for 6 h.

Neither feeding LSM nor implanting ewes with E\(_2\) altered \((P \geq 0.17)\) mucosal DNA or RNA concentrations, RNA:DNA, or protein:DNA ratios. However, there was a LSM by E\(_2\) interaction \((P = 0.02)\) in mucosal protein concentration. Although feeding LSM for 0 d and implanting ewes with E\(_2\) for 0 or 24 h were similar \((P = 0.24)\), protein concentrations decreased \((P = 0.01)\) after 24 h compared with ewes implanted for 6 h. Mucosal protein concentration of ewes fed LSM for 1 d and implanted with E\(_2\) for 24 h remained similar \((P = 0.41)\) compared with ewes implanted for 0 h; however, mucosal protein concentration increased \((P = 0.02)\) compared with ewes implanted for 6 h. There were no differences \((P \geq 0.47)\) observed in protein concentration when LSM was fed for 7 d. Implanting ewes with E\(_2\) for 6 h after feeding LSM for 14 d decreased \((P = 0.02)\) mucosal protein concentration compared with ewes implanted for 0 h. In addition, mucosal protein concentration tended \((P = 0.08)\) to be less when ewes were implanted for
24 h compared with ewes implanted for 0 h but was not different \((P = 0.53)\) from 6 h samples.

**IGF-I, T₃, and T₄**

To determine LSM and E₂’s effect on circulating hormone concentration, the change in IGF-I, T₃, and T₄ concentration was analyzed from each ewe from blood samples taken from 3 time-points: before LSM feeding; before E₂ implant; and before LSM slaughter (Table 5). Changes in IGF-I concentration from samples collected before feeding LSM to samples collected before E₂ implant were unaffected \((P = 0.15)\) by LSM feeding but tended \((P = 0.10)\) to be greater in ewes implanted for 6 h \((264 ± 88 \text{ ng/mL})\) than in ewes implanted for 24 h \((49 ± 88 \text{ ng/mL})\). There was a LSM × E₂ interaction \((P = 0.01)\) affecting the change in IGF-I concentration between implanting with E₂ and slaughter. When ewes were implanted with E₂ for 6 h, IGF-I concentration increased when ewes were fed LSM for 1 \((P = 0.05)\) or 7 d \((P = 0.002)\) whereas the change observed was not different from zero when LSM was fed for 0 \((P = 0.18)\) or 14 d \((P = 0.78)\). In contrast, IGF-I concentration decreased when ewes were implanted for 24 h and fed LSM for 1 \((P < 0.001)\), 7 \((P < 0.001)\), or 14 d \((P = 0.004)\). The magnitude of the increase in IGF-I levels was greater when ewes were implanted for 24 h and fed LSM for 1 \((P = 0.03)\) or 7 d \((P < 0.01)\) compared with ewes implanted for 24 h and fed LSM for 0 d. The change in IGF-I concentration in samples collected before feeding LSM to samples collected at time of slaughter was unaffected \((P = 0.45)\) by feeding LSM; however, implanting ewes with E₂ for 24 h increased \((P < 0.001)\) IGF-I concentration to a greater extent than ewes implanted for 6 h. The change observed in IGF-I concentration
Table 5. Effect of LSM feeding and implanting with E2 on change in plasma IGF-I and serum T3 and T4 concentration from pre-LSM feeding to slaughter, pre-E2 implant to slaughter, and pre-LSM feeding to slaughter in ewes fed LSM for 0, 1, 7, or 14 d and implanted with E2 for 6 or 24 h

<table>
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<th>7 d</th>
<th>14 d</th>
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<td>24 h</td>
</tr>
<tr>
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<td>336&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>-401&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>991&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-I (PE&lt;sub&gt;2&lt;/sub&gt; to Slt&lt;sup&gt;2&lt;/sup&gt;), ng/mL</td>
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</tr>
<tr>
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<tr>
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<td>1.7</td>
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<tr>
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</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt; (PF to PE&lt;sub&gt;2&lt;/sub&gt;), ng/mL</td>
<td>-74</td>
<td>91</td>
<td>-38</td>
<td>144</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt; (PE&lt;sub&gt;2&lt;/sub&gt; to Slt&lt;sup&gt;2&lt;/sup&gt;), ng/mL</td>
<td>110</td>
<td>47</td>
<td>-47</td>
<td>109</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt; (PF to Slt&lt;sup&gt;3&lt;/sup&gt;), ng/mL</td>
<td>-28</td>
<td>199</td>
<td>-66</td>
<td>302</td>
</tr>
</tbody>
</table>

*Within a row, means with different superscripts differ (P ≤ 0.05).
1 Change from pre-LSM feeding (PF) blood sample to pre-E2 implant (PE<sub>2</sub>) blood sample.
2 Change from pre-E2 implant (PE<sub>2</sub>) blood sample to slaughter (Slt) blood sample.
3 Change from pre-LSM feeding (PF) blood sample to slaughter (Slt) blood sample.
(-106 ± 135 ng/mL) when ewes were implanted for 6 h was not different from zero
($P = 0.45$) whereas an increase greater than zero ($P < 0.001$) of 1001 ± 135 ng/mL was
observed when ewes were implanted for 24 h.

Although the change in serum $T_3$ concentration from samples collected before
feeding LSM to samples collected before implanting ewes with $E_2$ remained unaffected
($P = 0.13$) by feeding LSM, $T_3$ concentration increased ($P = 0.04$) in ewes implanted with
$E_2$ for 24 h (5.0 ± 2.1 ng/mL) compared with ewes implanted for 6 h (-1.7 ± 2.1 ng/mL).
Change in serum $T_3$ concentration from samples collected before $E_2$ implant to samples
collected before slaughter was unaffected by LSM feeding ($P = 0.22$) and implanting
with $E_2$ ($P = 0.86$). From samples collected before implanting ewes with $E_2$ to samples
collected before slaughter, implanting ewes with $E_2$ for 24 h increased ($P = 0.01$) $T_3$
concentration 6.6 ± 1.9 ng/mL compared with ewes implanted for 6 h in which case $T_3$
concentration decreased by 2.3 ± 1.9 ng/mL.

Serum $T_4$ concentration followed trends similar to those observed for serum $T_3$
concentrations. From samples collected before feeding LSM to samples collected before
implanting with $E_2$, feeding LSM did not affect ($P = 0.91$) the change in $T_4$ concentration
whereas implanting ewes with $E_2$ for 24 h increased $T_4$ concentration by 183 ± 69 ng/mL
which was different ($P = 0.05$) from ewes implanted for 6 h where a decrease of
23 ± 69 ng/mL was observed. As was observed with $T_3$, neither feeding LSM nor
implanting ewes with $E_2$ affected ($P \geq 0.89$) the change observed in $T_4$ concentration
from samples collected before $E_2$ implant to samples collected at time of slaughter.
Change in serum $T_4$ concentration from samples taken before feeding LSM to samples
taken before slaughter was not altered ($P = 0.95$) by feeding LSM whereas implanting
ewes with E2 for 24 h increased serum T4 concentration by 219 ± 57 ng/mL, which was different ($P = 0.01$) compared with ewes implanted for 6 h where serum T4 concentration decreased by 6.4 ± 57 ng/mL.

In addition to changes in hormone concentration, absolute hormone concentration was also measured immediately before slaughter in all ewes (Table 6). Circulating concentrations of IGF-I, T3, and T4 as well as T4:T3 ratio from samples taken at slaughter were not affected ($P \geq 0.19$) by length of LSM feeding. Implanting ewes with E2 increased ($P < 0.001$) IGF-I concentration when ewes were implanted for 24 h (3039 ± 121 ng/mL) compared with ewes implanted for 6 h (2147 ± 121 ng/mL). Circulating IGF-I concentrations in ewes implanted for 0 h (2168 ± 121 ng/mL) were similar ($P = 0.90$) to concentrations observed in ewes implanted for 6 h but were less ($P < 0.001$) than levels observed in ewes implanted for 24 h. Similar to IGF-I, T3 concentrations increased ($P = 0.003$) in ewes implanted with E2 for 24 h (18.7 ± 1.8 ng/mL) compared with ewes implanted with E2 for 6 h (11.5 ± 1.8 ng/mL), whereas T3 concentration in ewes implanted for 24 h was similar ($P = 0.20$) to ewes implanted for 0 h (15.6 ± 1.8 ng/mL). Further, ewes implanted with E2 for 6 h had T3 concentrations that tended ($P = 0.10$) to be less than ewes implanted for 0 h. Implanting ewes with E2 did not alter ($P = 0.13$) T4 concentrations at slaughter. Although the T4:T3 ratio of ewes implanted with E2 for 24 h (67.3 ± 5.0) was similar ($P = 0.20$) to ewes implanted for 0 h (71.5 ± 5.0), the ratio increased ($P = 0.02$) when ewes were implanted for 6 h (88.4 ± 5.0) compared with ewes implanted for 0 h followed by a decrease ($P = 0.002$) when ewes were implanted for 24 h compared with ewes implanted for 6 h.
Table 6. Effect of LSM and E₂ on IGF-I, T₃, T₄, and T₄:T₃ ratio at time of slaughter¹

<table>
<thead>
<tr>
<th>Days fed LSM</th>
<th>0 d</th>
<th>1 d</th>
<th>7 d</th>
<th>14 d</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
<td>0 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Item</td>
<td></td>
<td></td>
<td></td>
<td>SEM</td>
<td></td>
</tr>
<tr>
<td>IGF-I, ng/mL</td>
<td>2030</td>
<td>2221</td>
<td>2472</td>
<td>2287</td>
<td>2123</td>
</tr>
<tr>
<td>T₃, ng/mL</td>
<td>14.4</td>
<td>13.2</td>
<td>15.5</td>
<td>15.1</td>
<td>12.8</td>
</tr>
<tr>
<td>T₄, ng/mL</td>
<td>955</td>
<td>1013</td>
<td>1053</td>
<td>1054</td>
<td>894</td>
</tr>
<tr>
<td>T₄:T₃</td>
<td>66.8</td>
<td>72.8</td>
<td>75.7</td>
<td>71.6</td>
<td>98.5</td>
</tr>
</tbody>
</table>

¹Treatments were days of linseed meal feeding (LSM; 0, 1, 7, and 14 d) and length of estradiol-17β exposure (E₂; 0, 6, and 24 h).
Discussion

This study has demonstrated that LSM is capable of both altering organ mass as observed in the decreased liver mass after 14 d of LSM feeding as well as interacting with E2 to alter organ mass as observed with the effects on uterine and duodenal mass. Although the impact of E2 on reproductive tissues has been well documented, the reported effects of E2 on visceral organ mass is limited. Magness et al. (1998) noted that in addition to increasing blood flow to reproductive tissues, blood flow to the small bowel was also increased in response to E2 administration in ovariectomized ewes indicating that these tissues are responsive to E2 and may therefore also respond to exposure to LSM.

Although plasma E2 concentration of ewes implanted for 6 h were different from ewes implanted for 24 h, this difference is thought to be inconsequential. Plasma E2 concentration for ewes implanted for both 6 and 24 h were supra-physiological compared with data from Kiyma et al. (2004) who reported E2 levels range from approximately 1 to 8 pg/mL in cycling ewes. We realize that these concentrations of E2 do not simulate those found in intact cyclic ewes; however, we feel that these conditions are adequate to study possible LSM and E2 interactions. In future studies, it may be beneficial to examine the possibility that LSM alters ovarian production or metabolic clearance rates of E2 (Pfeiffer et al., 2005) or other hormones (Ovesen et al., 1998; Van Pelt et al., 2003). At the levels observed in this study, we feel it can be assumed that E2 receptors in ewes implanted with E2 reached saturation and that difference in responses were due to either changes in E2 receptor number or modification of downstream effects of E2 receptor binding.

Although Rumsey et al. (1996) observed a decrease in liver mass (g/kg EBW) of steers implanted with a commercially available estrogenic implant (Synovex-S; 20 mg
estradiol benzoate and 200 mg progesterone) for 56 d, Hutcheson et al. (1997) observed an increase in liver mass (g/kg EBW) in steers implanted with Synovex-S for approximately 112 d. Our study showed a tendency for liver mass to increase when ewes were implanted with E2 and for liver mass to decrease when ewes were fed LSM. Although the implications of this are not yet clear, alterations in liver size may result in changes in basal metabolic requirements of the liver (Burrin et al., 1988; Burrin et al, 1990; Freetly and Ferrell, 1994a) and possibly metabolism of steroids by the liver via changes in blood flow (Freetly and Ferrell, 1994b; Wallace et al., 1997; Sangsritavong et al., 2002). It is not clear from DNA, RNA, and protein data obtained during this trial whether changes in liver weight in response to LSM were due to a decrease in cell number, cell size or some combination of the two; however, the tendency for a decrease in RNA:DNA ratio when LSM is fed indicates a possible decrease in liver cellular activity. This apparent decrease in liver activity in combination with LSM's ability to alter the liver's response to E2 (Figure 2) provide evidence that metabolic activity in the liver maybe altered through feeding LSM.

The uterotropic property of E2 is well established (Zondek, 1936; Kirkland et al., 1979; Reynolds et al., 1998). Reynolds et al. (1998) also reported a uterine weight increase associated with a decrease in DNA concentration of fresh tissue and a decrease in DM percentage in ovariectomized ewes exposed to E2 for 24 h. This effect was also observed in the current study. Feeding LSM for 1 d exacerbates the uterotropic activity of E2 but when LSM is fed for 7 or 14 d, the uterine response to E2 returns to that observed when LSM was fed for 0 d. Although feeding LSM in the absence of E2 did not alter uterine weights in our study, data obtained from studies in gilts (Ford et al., 2006) and rats (Whitten et al., 1992)
indicate that diets high in genistein, a phytoestrogen found in soy, increased uterine weight. Phytoestrogens, including those from soy as well as LSM, are selective estrogen response modulators (Nilsson et al. 2001). Although selective estrogen response modulators, including lignans, interact predominantly with the estrogen receptor, they induce effects that are different than the natural ligand and, in fact, can vary depending on the tissue being examined and the phytoestrogen under investigation (Dutertre and Smith, 2000).

In addition to reproductive tissues, the presence of estrogen receptors expressed within the intestine has been observed (Kawano et al., 2004). These receptors are functional in that they are able to induce physiological changes in the small intestine (Diaz et al., 2004; Chen et al., 2005). Pace et al. (2006) reported that intestinal mass was greater in sheep consuming diets abundant in subterranean clover compared with control sheep consuming diets abundant in Italian ryegrass. Analysis of the clover indicated that the predominant phytoestrogen present was genistein; however, several phytoestrogen compounds were present. This is in contrast with data from Chen et al. (2005) who found no differences in small intestinal mass in piglets fed genistein however, duodenal, jejunal, and ileal weights were not reported individually. In the current study, feeding LSM interacted with implanted E2 to affect only the duodenum whereas the jejunum and ileum remained unchanged due to either LSM or E2.

The effect that LSM had on the stomach complex mass was unexpected. It is currently unknown whether this is due to uniform changes in the entire stomach complex, or if feeding LSM influenced the change in mass of the rumen, reticulum, omasum, or abomasum individually since masses of the individual stomach complex organs were not
measured. Moreover, it is unknown if changes in mass of the stomach complex were due to
the direct effect of factors produced during fermentation or digestion within the stomach
complex itself, or if circulating metabolites influenced these changes. If LSM, by itself or
through interaction with circulating E₂, is capable of altering the growth of and mass of
visceral organs, then organ function (Diaz et al., 2004), digestive efficiency, and energy
expenditure (Ferrell, 1988) of organs of the digestive tract may also be affected. Further
research is needed to determine if the effects of feeding LSM and its interaction with E₂,
either in animals receiving an estrogenic implant or in intact, cyclic females, will contribute
to an improvement in gain efficiency and/or metabolic efficiency, or if changes in liver size
and small intestinal mass will have a positive or negative impact on animal production and
reproduction.

Individual variation of hormone parameters made interpretation of hormone
concentration difficult. Even marked differences, especially across treatments implanted
for 0 h and fed LSM for differing durations, were not detectable at time of slaughter.
Studies incorporating larger numbers of ewes may be required to interpret the effects of
LSM on hormone parameters. It is also not fully understood why, in blood samples taken
before implanting ewes with E₂, such a deviation exists between hormone concentrations
within a duration of LSM feeding for ewes implanted for 6 or 24 h. The only explanation is
that ewes implanted for 6 h were exposed to LSM for 18 h longer than those ewes
implanted for 24 h. However, this doesn’t seem plausible since LSM did not seem to have
an effect when fed without implanting ewes with E₂ (Table 6). Perhaps the best way to
evaluate the effects of LSM and E₂ on hormone concentration in this study is to interpret
the change in hormone concentration between blood samples (Table 5). Johnson et al.
(1996) noted an increase in circulating IGF-I when steers received Revalor-S implants (120 mg of trenbolone acetate + 24 mg of E2). In this study, ewes implanted with E2 for 24 h increased circulating IGF-I concentration between the time before implant and slaughter. Insulin-like growth factor-I is linked closely to growth (Florini et al., 1996; Owens et al., 1999) and it has also been established that circulating concentrations of IGF-I can be influenced by estrogens (Klotz et al., 2000; Leung et al., 2004). In this study, feeding LSM for 1, 7, and 14 d followed by implanting with E2 caused an increased response in that these ewes had greater circulating IGF-I concentrations than ewes fed LSM for 0 d. It is not clear, however, if this effect will continue if LSM feeding is extended or if ewes were implanted with E2 for greater than 24 h. Further experiments examining the dynamics of these changes would also be helpful in interpreting the effects of both feeding LSM and implanting with E2 on IGF-I concentration.

The thyroid hormones T3 and T4 affect liver function (Malik and Hodgson, 2002) and lipogenesis as well as metabolism (Yen, 2001). Only E2 appeared to alter T3 and T4 concentration (Tables 5 and 6). These results agree with data from Kahl et al. (1978) who found that steers implanted with Synovex-S had increased T4 levels 60 d after being implanted compared with steers not implanted. Davis et al. (1978) also noted that wethers implanted with diethylstilbestrol had greater levels of thyroid stimulating hormone whereas in vitro data from Miller et al. (1977) found that ovine pituitary cells cultured with E2 secreted greater levels of thyroid stimulating hormone.

It is evident that feeding LSM can influence body tissues as well as interact with E2 to influence these tissues. These data are limited however, because it does not accurately simulate conditions found in an intact animal, nor does it investigate long term effects of
feeding LSM. The competitive binding nature of phytoestrogens (Whitten et al., 1992) allow for the possibility that when systemic concentration of E2 is reduced to levels found in nature, fewer E2 receptors would be occupied by E2 and thus available to bind lignans. In addition to the direct effect on E2 receptor binding, compounds altering the level of SHBG can also influence the number of E2 receptors occupied by either E2 or lignans. Adlercreutz et al., (1987) noted a positive correlation between excretion of lignans and plasma SHBG and Mousavi and Adlercreutz (1993) found that genistein induced the production of SHBG in vitro. Further research is needed in the effects of feeding LSM on the reproductive and digestive tracts. How LSM affects these characteristics when fed for extended time periods as well as the interactions of feeding LSM with the presence of endogenous E2 as in the case of intact females needs to be determined as well as LSM’s effects on male, both intact and emasculated, animals.

**Literature Cited**


CHAPTER 3
LINSEED MEAL AND ESTRADIOL-17β ALTERS CELLULARITY,
ANGIOGENIC FACTORS, AND VASCULARITY OF THE JEJUNUM


Abstract

Cellular proliferation; vascularity; and the expression of the angiogenic factors vascular endothelial growth factor (VEGF), VEGF receptor-1 (FLT), VEGF receptor-2 (KDR), fibroblast growth factor (FGF), FGF receptor 2 IIIc (FGFR), angiopoietin (ANG)-I, ANG-2, ANG receptor (Tie-2), endothelial nitric oxide synthase (eNOS), and soluble guanylate cyclase (sGC) were evaluated in 48 ovariectomized ewes (54.6 ± 1.1 kg) fed a diet containing 12.5% LSM for 0, 1, 7, or 14 d and implanted with E2 for 0, 6, or 24 h before tissue collection. The phytoestrogen SDG is found in high concentrations in LSM and has been observed to elicit estrogenic effects. There was a LSM × E2 interaction (P = 0.003) on the jejunal cellular proliferation index. Jejunal cellular proliferation increased (P < 0.002) in ewes not fed LSM and implanted with E2 for 6 or 24 h compared with ewes implanted for 0 h, but did not increase when ewes were fed LSM for 1, 7, or 14 d. Neither feeding LSM nor implanting ewes with E2 altered vascular area density (P > 0.75) or vascular surface area (P > 0.29) of the jejunal villi. Expression of mRNA for the angiogenic factors VEGF, FGF, FGFR, ANG-I, ANG-II, and Tie-2 was not altered (P > 0.33) by feeding LSM or implanting ewes with E2. Implanting ewes with E2 for 6 h increased (P = 0.04) eNOS expression compared with ewes implanted for 0 h. Feeding
LSM and implanting ewes with E_2 interacted to alter mRNA expression of FLT \( (P = 0.04) \), KDR \( (P < 0.001) \), and sGC \( (P = 0.04) \). When LSM was fed for 1 d, but not 0, 7, or 14 d, expression of FLT mRNA decreased \( (P < 0.03) \) when ewes were implanted with E_2 for 24 h compared with ewes implanted for 0 or 6 h. Expression of KDR mRNA was suppressed in ewes fed LSM for 1 d \( (P = 0.03) \) or 7d \( (P = 0.0007) \) and implanted with E_2 for 24 h compared with ewes implanted for 0 h. When LSM was fed for 14 d, implanting ewes for 6 h increased \( (P = 0.04) \) KDR expression compared with ewes implanted for 0 h. Ewes fed LSM for 0 and 1 d experienced an increase in sGC mRNA expression when implanted for 6 h \( (P = 0.001) \) compared with ewes implanted for 0 h. When implanted for 24 h, levels were similar \( (P = 0.80) \) to those observed when ewes were implanted for 0 h. Expression of sGC was not altered by E_2 when LSM was fed for 1, 7, or 14 d \( (P > 0.11) \). Further research is needed to investigate LSM’s effect on cellular proliferation and angiogenesis in other tissues especially in organs of the female reproductive tract and the placenta.

**Introduction**

In ovariectomized ewes, E_2 exposure for 24 h increased cellular proliferation, microvascular density, and angiogenic factor expression of the endometrial epithelium (Reynolds et al., 1998a,b). In as much as E_2 is able to elicit a response in cellularity and angiogenesis within the endometrium, it is conceivable that any tissue expressing receptors to E_2 could also be altered when exposed to E_2 or E_2 agonists or antagonists. Functional E_2 receptors are present in intestinal epithelial cells (Thomas et al., 1993). Chen et al. (2005) reported that feeding the phytoestrogen genistein, a compound found in soy products and
whose metabolites are capable of interacting with the estrogen receptor, altered the cellular proliferation rate of intestinal crypt cells of piglets.

The inclusion of flaxseed (*Linum unisatissimum*) or LSM, a byproduct of flaxseed processing, in animal diets offers several benefits to livestock producers. Increased conception rates have been observed in dairy cattle fed flax or flax products (Petit et al., 2001; Petit et al., 2004) and improved feedlot performance and carcass quality grades have been observed in beef cattle (Maddock et al., 2006). The phytoestrogen SDG, found in flaxseed and LSM, can be metabolized by intestinal microflora (Axelson and Setchell, 1981) and possibly gastrointestinal enzymes (Day et al., 2000) to the mammalian lignans enterodiol and enterolactone (Axelson et al., 1982; Rickard et al., 1996; Begum et al., 2004) which are able to interact systemically with estrogen receptors. The objectives of this study were to determine how the length of LSM feeding and E2 exposure affect cellularity and vascularity of the jejunum in addition to the expression of the angiogenic factors vascular endothelial growth factor (VEGF), VEGF receptor-1 (FLT), VEGF receptor-2 (KDR), fibroblast growth factor (FGF), FGF receptor 2 IIIc (FGFR), angiopoietin (ANG)-1, ANG-2, ANG receptor (Tie-2), endothelial nitric oxide synthase (eNOS), and soluble guanylate cyclase (sGC) in ovariectomized ewes.

**Materials and Methods**

**Animals and Treatments**

All experimental protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee before the initiation of the research. For complete descriptions of animals, diets, and treatments, please refer to Chapter 2. Briefly, forty-eight multiparous ewes (54.6 ± 1.1 kg initial BW) were ovariectomized via
midventral laparotomy (Reynolds et al., 1998a), and allowed to recover for at least 28 d before treatments were initiated. After ovariectomy, ewes were fed an LSM free diet (Chapter 2) until initiation of treatment. The feeding of an LSM free diet following ovariectomy was carried out to ensure that any circulating endogenous estrogens as well as dietary phytoestrogens were cleared from the body before treatments were begun. Ewes were fed to meet their requirement for maintenance for each pen based on their metabolic body weight (NRC, 1985). Ewes were weighed every two weeks and had ad libitum access to water and were fed once daily at 0800 throughout the experiment. Immediately before initiation of treatment, ewes were moved to individual pens measuring 1.52 x 1.87 m. Ewes were weighed and stratified into blocks based on BW (i.e. 12 lightest ewes were assigned to block 1, next 12 lightest ewes were assigned to block 2, etc.). Once stratified, ewes within a block were assigned randomly to each treatment group. Treatments were arranged as a 3 x 4 factorial. Main effects consisted of length feeding a diet containing 12.5% LSM (as fed basis; Chapter 2) and length of exposure to E2 via subcutaneous implant.

The LSM diet was designed to be phytoestrogen free with the exception of the inclusion of 12.5% LSM. Laboratory analysis of the LSM used to formulate the LSM diet indicated the SDG content of the LSM diet to be 14.53 mg/g of feed on a DM basis. Control and LSM diets were formulated to be similar in protein (13.7 and 13.6% CP, respectively) and energy (1.796 and 1.794 kcal of NE\textsubscript{m}, respectively). Both diets were pelleted and fed to meet the net energy and CP requirements for maintenance based on ewe metabolic BW (56 kcal/kg\textsuperscript{0.75}; NRC, 1985). Ewes were either fed the control diet throughout the experiment until tissues were collected or, after being fed the
phytoestrogen-free diet for at least 1 month, were fed the LSM diet for 1, 7, or 14 d before
tissue collection. Ewes within each duration of LSM feeding were implanted with E₂ for 0,
6, or 24 h before tissue collection. Thus, each treatment group consisted of 4 ewes. Ewes
receiving E₂ implants were locally anesthetized and implanted subcutaneously in the
axillary region as validated by Johnson et al. (1997a). Implanted ewes each received two
Silastic implants (3.35 mm i.d. × 4.65 mm o.d. × 15 mm length; Dow Corning, Midland,
MI) each containing 50 mg of E₂ (Sigma-Aldrich, St. Louis, MO). After implantation, the
incision was closed with surgical staples and a topical antibiotic was applied to the incision
site. The implants remained until tissue collection at which time their presence was
confirmed.

**Tissue Collection**

Exactly one h before tissue collection, ewes were injected via jugular venipuncture
with BrdU (Aldrich, Milwaukee, WI; 5 mg/kg BW; Zheng et al., 1996; Johnson et al.,
1997a, b). At time of tissue collection, ewes were stunned via captive bolt and
exsanguinated. The viscera were removed from the body cavity and the jejunum was
located as previously described in Chapter 2 (Scheaffer et al., 2004; Reed et al., 2007).
Briefly, the distal 300 cm of the jejunum was divided into two 150-cm sections. The cranial
150-cm section was carefully removed with mesentery and vasculature intact, placed in
PBS, and immediately transported to the lab. To obtain the mass of the entire jejunum, the
remaining jejunum was stripped of its contents, separated from the mesentery, and
weighed. The mass of the caudal 150-cm section of the jejunum was doubled to account for
the cranial 150-cm section that was previously removed for perfusion.
Approximately 10 cm of the proximal end of the cranial 150-cm section of jejunum was subsampled and snap-frozen in liquid nitrogen and stored at -70°C for later mRNA quantification. The remaining section of jejunum was fixed via vascular perfusion by methods adapted from Soto-Navarro et al. (2004) and Reed et al. (2007). Briefly, hemostats were used to isolate approximately two vascular arcades within the mesentery. A primary branch of the mesenteric artery was then dissected from the mesentery and catheterized using polyethylene tubing (PE-160; o.d. = 1.56 mm, i.d. = 1.14 mm; Becton Dickinson, Sparks, MD). Once catheterized, vessels were flushed with approximately 10 mL of PBS followed by perfusion with approximately 3 mL of Evans blue dye (0.05% in PBS, wt/vol; Sigma Chemical Co., St. Louis, MO) to allow visualization of the vascular area being perfused. Once the field was visualized, the Evans blue was flushed from the vessels with 10 to 20 mL of PBS. After flushing with PBS, the jejunum was perfuse-fixed with 10 to 15 mL of Carnoy’s solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid). Following perfuse-fixing, the mesentery was carefully trimmed from the jejunum and the jejunum was immersed in Carnoy’s solution. Following fixation, the tissues were embedded in paraffin, sectioned to 4 µm, and affixed to glass slides for staining.

Tissue Analysis

Jejunal mRNA expression of VEGF, FLT, KDR, eNOS, FGF, FGF-R2 IIic, ANG-1, ANG-2, Tie-2, sGC was determined using quantitative RT-PCR as adapted from Redmer et al. (2005) and Vonnahme et al. (2006) following capillary electrophoresis of total cellular RNA using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE) to determine quantity and quality of extracted total cellular RNA.
Perfuse-fixed jejunal tissues were sectioned to 4 µm and affixed to glass slides. Two staining protocols were used for jejunal tissues from each ewe to determine vascularity and cellular proliferation. Slides evaluated for cellular proliferation were deparaffinized in graded ethanol, rinsed in distilled water, bathed in 3% H₂O₂ for five min, rinsed in distilled water, bathed in 2 N HCl for 30 min, rinsed with distilled water, and then bathed in PBS buffered with Triton X-100 for 10 min followed by a 10 min bath in PBS without Triton X-100. At this time, the slides were moved to a Dako Autostainer (Dako; Carpenteria, CA). The following staining protocol was programmed into the autostainer: (unless it is specified that the antibody or reagent was rinsed from the slide, it can be assumed that the reagent was blown from the slide before the next step rather than completely rinsed from the slide): TBS rinse; 10 min protein block (Serum free protein block; Dako); 60 minute incubation with primary antibody (Envision mouse anti-BrdU diluted 1:150 with antibody diluent; Dako); TBS rinse; 30 minute incubation with rabbit anti-mouse labeled polymer (Dako); TBS rinse and TBS was blown from the slide; 5 min rinse in PBS and PBS was blown dry; 10 min incubation with Vector SG substrate (Vector Labs, Burlingame, CA). The slides were then removed from the autostainer and rinsed in distilled water before being bathed in nuclear fast red for 15 min. This was followed by a final rinse, passage through graded ethanol, and mounted with coverslips. Slides evaluated for vascularity were prepared as described by Reed et al. (2007). Briefly, slides were deparaffinized via graded ethanol, rinsed in distilled water and stained with periodic acid Schiff’s reagent for approximately 10 min.

Digital images of tissues were collected for both vascularity and proliferation data using a Nikon DXM 1200 digital camera (Fryer, Chicago, IL) and a image analysis
software package (Image-Pro Plus, version 5.0; MediaCybernetics, Inc., Silver Spring, MD). Proliferation was determined by image analysis of BrdU stained cells in the crypt region of 6 fields for each ewe. The percentage of BrdU stained nuclei of all nuclei present in the crypt region was determined to obtain a proliferation index. Total cells in the jejunum were determined as the quotient of jejunal DNA content and $6.6 \times 10^{-12}$ g DNA/cell (Baserga, 1985). The proliferation index was then used to determine the number of proliferating and non-proliferating cells. The middle third of individual jejunal villi were used to determine measurements of jejunal vascularity. Measures of vascular area density and vascular surface area were collected from 10 jejunal villi per ewe and determined via image analysis.

**Statistics**

Data for ewe jejunal cellularity, cellular proliferation, vascularity, and angiogenic factor expression were analyzed as a $3 \times 4$ factorial in a randomized complete block design using the PROC MIXED procedures of SAS (SAS Inst. Inc., Cary, NC). Model statement included effects of block (based on initial weight), length of LSM feeding, length of E$_2$ exposure, and the interaction of LSM feeding and E$_2$ exposure. Fixed variables were length of LSM feeding, length of E$_2$ exposure, and block. The diagonal covariate structure was used. Data are presented as least squares means ± SEM. Mean separations were performed via LSD which were protected by an overall treatment $F$-test at $P = 0.05$. Differences were considered significant if $P \leq 0.05$ unless otherwise stated.

**Results**

Measurements of ewe weight, visceral organ weight, mucosal percentage of the jejunum, and DNA, RNA, and protein concentration and content of the jejunum have been
previously reported (Chapter 2). Neither feeding LSM nor implanting ewes with E2 affected the total number of cells within the crypt region of the jejunum (Table 6; \( P = 0.21 \) and \( P = 0.65 \), respectively) or the number of non proliferating cells (\( P = 0.22 \) and \( P = 0.79 \), respectively); however, there was a LSM \( \times \) E\textsubscript{2} interaction (\( P = 0.003 \)) on the cellular proliferation index within the crypt region of the jejunum and a resultant tendency (\( P = 0.09 \)) for a LSM \( \times \) E\textsubscript{2} interaction on the number of proliferating cells in the jejunum.

When ewes were fed LSM for 0 d, implanting ewes with E\textsubscript{2} for 24 h increased the labeling index (BrdU labeled cells/total cells within the crypt region of the jejunum) when compared with ewes implanted with E\textsubscript{2} for 0 or 6 h (\( P = 0.002 \) and \( P < 0.001 \), respectively). However, when LSM was fed for 1, 7, or 14 d the increase in the labeling index seen in response to implanting ewes with E\textsubscript{2} for 24 h was not observed. When evaluating the total number of proliferating cells, feeding LSM and implanting ewes with E\textsubscript{2} tended (\( P = 0.09 \)) to interact and resulted in patterns similar to those observed when proliferation is expressed as a percentage of total cells.

There were no differences observed for any measurement of vascularity within the jejunum (Table 7). Neither feeding LSM nor implanting ewes with E\textsubscript{2} altered measurements of vascular area density (\( P = 0.77 \) and \( P = 0.75 \), respectively) or vascular surface area (\( P = 0.95 \) and \( P = 0.29 \), respectively). Although mRNA expression of VEGF (Table 8) remained unaltered due to feeding LSM (\( P = 0.51 \)) or implanting with E\textsubscript{2} (\( P = 0.36 \)), mRNA expression of its receptors, KDR and FLT, were altered as a result of an interaction of feeding LSM and implanting with E\textsubscript{2} (\( P = 0.0002 \) and \( P = 0.04 \), respectively). In the case of KDR, when ewes were implanted with E\textsubscript{2} for 0 h, feeding LSM for 1 and 14 d decreased (\( P = 0.05 \) and \( P = 0.0005 \), respectively) mRNA expression whereas feeding
Table 7. Effects of feeding LSM and implanting ewes with E2 on percentage cellular proliferation as well as the number of cells, number of proliferating cells, number of non-proliferating cells within the crypt region of the jejunum as well as the vascular area density and vessel surface area in the jejunal villi

<table>
<thead>
<tr>
<th>Item</th>
<th>Days fed LSM</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d</td>
<td>1 d</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>6 h</td>
</tr>
<tr>
<td>Labeling index: %</td>
<td>0.86</td>
<td>2.37</td>
</tr>
<tr>
<td>Total cells: 10^6</td>
<td>1713</td>
<td>1455</td>
</tr>
<tr>
<td>Proliferating cells: 10^6</td>
<td>17.9</td>
<td>25.5</td>
</tr>
<tr>
<td>Non-proliferating cells: 10^6</td>
<td>1698</td>
<td>1419</td>
</tr>
<tr>
<td>Vascular area density</td>
<td>13.1</td>
<td>14.2</td>
</tr>
<tr>
<td>Vascular surface area</td>
<td>89.8</td>
<td>95.4</td>
</tr>
</tbody>
</table>

^aWithin a row, means with different superscripts differ (P ≤ 0.05).

^1As determined by immunohistochemistry.

^2As determined by immunohistochemistry and quantification of tissue DNA concentration.
Table 8. Effects of feeding LSM and implanting ewes with E2 on jejunal expression of angiogenic factors vascular endothelial growth factor (VEGF), fms-like tyrosine kinase (FLT; VEGF receptor-1), kinase insert domain-containing receptor (KDR; VEGF receptor-2), fibroblast growth factor (FGF), fibroblast growth factor receptor 2 IIIc (FGF-R2 IIIc), angiopoietin I (ANG-I), angiopoietin II (ANG-2), angiopoietin receptor (Tie-2), endothelial nitric oxide synthase (eNOS), and soluble guanylate cyclase (sGC).

<table>
<thead>
<tr>
<th>Item</th>
<th>Days fed LSM</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d</td>
<td>1 d</td>
</tr>
<tr>
<td></td>
<td>Hours exposed to E2</td>
<td>Hours exposed to E2</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.075 0.092 0.114</td>
<td>0.095 0.100 0.043</td>
</tr>
<tr>
<td>FLT</td>
<td>0.007a 0.006b 0.009b</td>
<td>0.013b 0.019b 0.005d</td>
</tr>
<tr>
<td>KDR</td>
<td>0.147a 0.117ab 0.092ab</td>
<td>0.089b 0.054c 0.009c</td>
</tr>
<tr>
<td>FGF</td>
<td>0.105 0.389 0.253</td>
<td>0.195 0.199 0.148</td>
</tr>
<tr>
<td>FGFR</td>
<td>0.098 0.230 0.166</td>
<td>0.085 0.261 0.216</td>
</tr>
<tr>
<td>ANG-I</td>
<td>0.018 0.030 0.031</td>
<td>0.019 0.040 0.030</td>
</tr>
<tr>
<td>ANG-II</td>
<td>0.0010 0.0024 0.0015</td>
<td>0.0030 0.0032 0.0013</td>
</tr>
<tr>
<td>Tie-2</td>
<td>0.073 0.145 0.149</td>
<td>0.085 0.146 0.100</td>
</tr>
<tr>
<td>eNOS</td>
<td>0.112 0.271 0.233</td>
<td>0.119 0.333 0.165</td>
</tr>
<tr>
<td>sGC</td>
<td>0.150a 0.503b 0.173c</td>
<td>0.081a 0.255a 0.162c</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\)Within a row, means with different superscripts differ (P ≤ 0.05).

\(^1\)Values are arbitrary units.
LSM for 7 d increased \((P = 0.02)\) expression compared with ewes fed LSM for 0 d. Additionally, implanting ewes with \(E_2\) did not alter \((P > 0.10)\) mRNA expression when LSM was fed for 0 d but decreased expression with 24 h \(E_2\) exposure when ewes were fed LSM for 1 \((P = 0.03)\) and 7 d \((P = 0.0007)\). In contrast, mRNA expression of KDR increased \((P = 0.04)\) when ewes were implanted with \(E_2\) for 6 h when LSM was fed for 14 d.

Expression of FLT mRNA was not altered in response to \(E_2\) when LSM was fed for 0 \((P > 0.66)\), 7 \((P > 0.08)\), or 14 d \((P > 0.30)\) but decreased when ewes were implanted for 24 h compared with ewes implanted for 0 or 6 h \((P = 0.03\) an \(P = 0.004\), respectively) when LSM was fed for 1 d. Feeding LSM and implanting with \(E_2\) also interacted \((P = 0.04)\) to alter the expression of sGC. Implanting ewes with \(E_2\) for 6 h resulted in an increased \((P = 0.001)\) sGC mRNA expression when LSM was fed for 0 d compared with ewes implanted for 0 h. When ewes were implanted with \(E_2\) for 24 h, expression of sGC mRNA returned to levels similar \((P = 0.80)\) to those observed when ewes were implanted for 0 h. Feeding LSM for 1, 7, or 14 d resulted in sGC levels that were unaltered \((P > 0.11, P > 0.54,\) and \(P > 0.54,\) respectively) due to duration of \(E_2\) implant.

Implanting ewes with \(E_2\) tended \((P = 0.10)\) to alter expression of eNOS mRNA. Expression of eNOS mRNA was greater \((P = 0.04)\) in ewes implanted with \(E_2\) for 6 h \((0.218 \pm 0.030 \text{ ng})\) compared with ewes implanted for 0 h \((0.132 \pm 0.025)\). Expression of eNOS mRNA after 24 h of \(E_2\) \((0.182 \pm 0.25)\) was similar to levels observed when ewes implanted for 0 \((P = 0.18)\) and 6 h \((P = 0.36)\).
Discussion

Although phytoestrogens have been shown to alter cellular proliferation in the digestive tract (Chen et al., 2005), this is the first study in which LSM has been shown to interact with E₂ to affect cellular proliferation in the jejunum (Table 7). Greater indices of cellular proliferation in response to implanting ewes with E₂ (Reynolds et al., 1998a) or following estrus in intact, cyclic ewes (Johnson et al., 1997b) have been previously reported in uterine tissue. The results from the current study are similar to those which are observed in ovine uteri either following estrus in cycling ewes at which time E₂ levels are elevated or 24 h after implanting ewes with E₂ in ovariectomized ewes (Johnson et al., 1997b; Reynolds et al., 1998a).

Magness et al. (1998) observed an increase in blood flow to the small bowel of ovariectomized ewes following 10 d of E₂ infusion but did not observe a change after 120 min of E₂ infusion. It is therefore not surprising that changes in vascularity measurements were not observed after only 6 or 24 h of exposure to E₂ in the current study (Table 7). Perhaps vascularity measurements in the jejunum would be detectable if E₂ exposure were extended for a longer period. Although vascularity measurements were also not altered following LSM feeding periods of up to 14 d, further research may be necessary to determine the effects of feeding LSM to subjects exposed to E₂ for prolonged periods. Kruse et al. (1997) observed an inhibition of FGF induced ocular neovascularization in rabbits receiving a subconjunctival injection of genistein. In the current study, the increase in labeling index following exposure to E₂ was ablated when LSM was fed. Perhaps feeding LSM would similarly ablate an increase in blood flow to the small bowel as observed by Magness et al. (1998) in response to prolonged E₂ exposure.
The inability of E2 exposure to alter VEGF expression in the jejunum is in contrast to data which uterine VEGF expression is elevated in cyclic ewes after 4, 8, and 24 h of being implanted with E2 (Reynolds et al., 1998b). Suzuma et al. (1999) also noted an increase in VEGF mRNA expression in cultured bovine retinal endothelial cells 24 h after E2 exposure. This difference in VEGF expression patterns in response to E2 is likely due to the difference in tissue type. Angiogenesis is common in wound healing and developing tissues as well as cyclically in organs of the reproductive tract, but not common in adult tissue (Fotsis et al., 1993, Reynolds and Redmer, 1998). It is therefore feasible that non-reproductive tissues may respond differently in angiogenic factor expression in response to E2 than reproductive tissues and, in fact, uterine tissue obtained from ewes in the current study responded similarly to previous studies (Reynolds et al., 1998b) exhibiting an increased in caruncular VEGF mRNA expression after 6 h of being implanted with E2 irrespective of the length of time they were fed LSM (our unpublished data).

Suzuma et al. (1999) noted an increase in KDR mRNA expression in bovine retinal endothelial cells after exposure to E2 for 6 to 24 h. This was not observed in the present study in jejunal tissue when LSM was fed for 0 d. Furthermore, a decrease in KDR mRNA expression in response to exposure to E2 for 24 h was observed when LSM was fed for 1 or 7 d whereas implanting ewes fed LSM for 14 d increased expression. Expression patterns of VEGF mRNA due to feeding LSM were also peculiar with expression increasing when LSM was fed for 7 d but decreasing when LSM was fed for 1 or 14 d in ewes not implanted with E2. Although FLT mRNA expression was altered less by LSM and E2, it did respond similarly when LSM was fed for 1 d with a decrease in expression in ewes implanted for 24 h. These data indicate that in the jejunal angiogenesis may be altered by altered expression
of FLT and KDR rather than by altering expression of VEGF. These data also conflict somewhat with views of phytoestrogens as being mainly antiangiogenic (Fotsis et al., 1993; Kruse et al., 1997; Dubey et al., 2000) Furthermore, these data also indicate that phytoestrogens are capable of ablating the angiogenic properties of E2 (Cullinan-Bove and Koos, 1993; Suzuma et al., 1999)

Weiner et al. (1994) observed an increase in eNOS expression in response to E2. Rosselli et al. (1995) also observed an increase in circulating levels of nitric oxide in postmenopausal women supplemented with E2. These reports agree with the present study where 6 h of E2 exposure caused an increase in eNOS expression. Although feeding LSM did not appear to alter eNOS expression, when fed for 1, 7, or 14 d, it did suppress an increase in sGC mRNA expression that was observed after 6 h of E2 exposure when LSM was fed for 0 d. The present study conflicts with data from Krumenacker et al. (2001) who reported that sGC mRNA expression decreased in immature rat uteri 3 h after an injection of E2 whereas expression in the lung, liver, and vascular tissue remained unchanged. These differences may be tissue specific or due to the immaturity of the rats.

This study confirmed that LSM and E2 interact to alter cellular proliferation within the crypt region of the jejunum. To our knowledge, this is the first study showing that E2 is capable of altering cellular proliferation within the gut. The implication of these findings are yet unknown, although important as estrogen levels increase dramatically in animals during estrus and pregnancy. Shurson et al. (2003) reported that pregnant sows gained weight more efficiently than non-pregnant sows during gestation. Scheaffer et al. (2004) reported that both vascularity percentage and total microvascular volume of the jejunum were increased in pregnant ewes compared with non-pregnant ewes indicating that jejunum
increases absorptive capacity in response to the increasing nutritional demands of pregnancy. The physiological signal for the small intestine to do so is not known. Perhaps the elevated levels of E\textsubscript{2} observed during pregnancy act as a signal for increased nutrient absorption in small intestine. The present study did not investigate what effect prolonged exposure to E\textsubscript{2} (longer than 24 h) would have on cellular proliferation or vascularity of the gut. These parameters require further investigation in addition to its interaction with LSM as it is evident from the present study that LSM is capable of interacting with E\textsubscript{2}.

Although we did not detect any alterations in vascularity of the intestinal villi, mRNA expression of eNOS was increased after 6 h of E\textsubscript{2} implant and FLT, KDR, and sGC were altered by an interaction of E\textsubscript{2} and LSM. Although the present study examined the expression of angiogenic factor mRNA, it should be noted that this may not correlate with phenotypic expression of the gene product (Rehfeld, 1998). Further research may be useful to determine if the changes in mRNA expression due to interactions of LSM and E\textsubscript{2} resulted ultimately in altered protein expression. Furthermore, the present study focused on the jejunum of adult, ovariectomized ewes. Since angiogenesis is, for the most part, restricted to the female reproductive organs in adult organisms (Fotsis et al., 1993; Reynolds and Redmer, 1998), it is necessary to determine how LSM affects angiogenesis in the ovary, follicle, corpus luteum, and uterus of the adult female in addition to the placenta of the pregnant female. Additionally, LSM’s effect on angiogenesis of the developing conceptus and adolescent also needs to be examined.

**Literature Cited**


CHAPTER 4

GENERAL CONCLUSIONS

The novel findings in the present study, including LSM's ability to decrease the mass of the liver, and LSM's interaction with E2 to alter duodenal and uterine weight, cellularity in the jejunum, and the expression of the angiogenic factors FLT, KDR, and sGC, provide a number avenues for future research in the area of phytoestrogens and lignans. Future research comparing not only length of exposure to LSM, but also concentration should be examined. Many of the biological effects of other phytoestrogens, including genistein, are considered estrogenic at low concentrations but antiestrogenic at high concentrations. Additionally, the use of intact, cyclic animals should also be considered in an effort to more closely simulate the biology and physiology of these animals as it would be in the pasture or feedlot.

Much of the biological effects of SDG and phytoestrogens, in general, are attributed to their ability to inhibit angiogenesis. Although the present study did not observe an effect on vascularity in the jejunum in response to either E2 or LSM, it is important to note that angiogenesis is not common in normal adult tissues. If exposure to LSM inhibited angiogenesis of prenatal or neonatal offspring, the consequences could be detrimental. As the tissues of the female reproductive tract do normally experience angiogenesis as does the placenta, understanding how SDG affected these tissues would be useful in understanding how feeding LSM might alter reproductive traits such as cyclicity; return to estrus following postpartum anestrus; conception rates; and, in conjunction with the placenta, development of the feto-maternal exchange of nutrients during pregnancy.
Alterations in jejunal vascularity during pregnancy in the dam are presumably an effort to increase nutrient absorptive capacity to facilitate increasing energy demands of the dam and fetus during pregnancy. Investigating the effects of LSM on the angiogenesis of the jejunum during pregnancy could yield valuable evidence as to the effects of LSM on nutrient availability to the dam and offspring during pregnancy.

Although some phytoestrogens such as genistein have been extensively researched, relatively few studies have focused on SDG. Currently, many generalizations are made concerning phytoestrogens and their effects; however, it is obvious that not all phytoestrogens react identically and the thorough investigation of SDG and LSM are necessary.