Clinical Changes in Ovariectomized Ewes Exposed to Phytoestrogens and 17β-Estradiol Implants (43838)

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Abstract. **Eight Swedish Finewool Landrace ewes, ovariectomized 5 months earlier and kept on nonestrogenic hay, were each fed 3.5 kg red clover silage, corresponding to 6.1 g phytoestrogens (of which 3.5 g was formononetin) per day, for 14 days in November (short days). In January (short days), two groups (3 each) of these ewes received one or two 17β-estradiol sc implants. In May (long days), one of two new groups (4 each) of these ewes was reexposed to phytoestrogens for another 14 days while the other served as a control. Physical examination of ewes for changes in reproductive organs was carried out two or three times per week during each feeding/treatment, and continued until observed changes disappeared. Clinically significant changes occurred in the reproductive organs of ewes fed red clover. Vulva color changed from pale to pink and red, and there were enlargements of the vulva, uterus, and udder. In addition, teat length and circumference increased, and secretion of milky fluid began. These changes were similar, but more pronounced during treatment with 17β-estradiol, particularly teat circumference. The changes in vulva were more dramatic in May than in November and resembled those observed in ewes treated with estradiol. Our data show that a daily intake of 3.5 g formononetin for 14 days caused the increase of teat size and changes in the color of the vulva and in uterus weight in ovariectomized ewes.** [P.S.E.B.M. 1995, Vol 208]

The presence of estrogenic substances in plants was first demonstrated in the 1920s (1, 2). Later, such substances were reported to cause reproductive disorders in sheep grazing subterranean clover in Australia (3). Genistein and other isoflavones, daidzein, biochanin-A, and formononetin were isolated from clover and proved to be the cause of the disorders (1). The estrogenic activities of forage plants have been reported from various parts of the world (4, 5, 6, 7). It is known that the relative activity of individual isoflavones may vary depending on the strain as well as the species of animals (8) owing to inter- (2) and intraspecific variation in the metabolism of isoflavones (9). Formononetin is the most important isoflavone causing reproductive disorders in sheep (10) through its main stable metabolite, equol.

This paper presents the clinical responses of ovariectomized Swedish Finewool Landrace ewes exposed either to red clover silage or to 17β-estradiol.

Materials and Methods

Experimental Animals. Eight Swedish Finewool Landrace ewes, all of which had lambed in January, were ovariectomized in May through a mid-ventral laparotomy under general anaesthesia. The ewes were kept on pasture until September. They were then moved indoors and kept in individual, adjacent boxes under natural light conditions until the end of the experiment. The ewes were given *ad libitum* access to nonestrogenic hay (mainly Timothy grass) and 100 g of concentrate (39% barley, 39% oats, 11% soya, 7% rapeseed, and 4% other additions) per day except during experimental feeding periods. They had free access to mineral licks and good drinking water at all times.

Phytoestrogens. Red clover (*Trifolium pratense*)

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silage was used as the source of plant estrogens. High performance liquid chromatography was used to determine the estrogen content of the feed samples (11).

17β-Estradiol Implants. Silastic tubes (6 cm long, 0.335 cm i.d., 0.465 cm o.d.; Dow Corning Medical, Midland, MI) were filled with 17β-estradiol crystals (Sigma Chemical Co., St. Louis, MO) and sealed at both ends with silastic Medical adhesive type A (Dow Corning, Midland, MI). It has previously been reported that the release rate of 17β-estradiol from this size implants is 45 μg/day (12). Prior to use, implants were incubated in 100 ml normal saline for 24 hr at room temperature, under constant agitation to avoid a transient peak in plasma estradiol after insertion. Implants were introduced subcutaneously in the axillary region of ewes while anaesthetized by xylazine (Rompun® vet.; Bayer Sverige AB, Göteborg, Sweden) and removed after 14 days.

Experimental Protocol. At the end of October, all ewes (mean body weight of 64 kg) were pretreated for 6 days with feed consisting of incremental increases (20%, 30%, 50%, 50%, 75%, 75%) of red clover silage, to allow the rumen microbial populations to adapt. After the final pretreatment, 24 hr later (Day 0), the ewes were fed 3.5 kg of 100% red clover silage for 14 days (about 8 hr of daylight). Test samples were taken, the ewes were acquamated to a nonestrogenic diet by gradually reducing the red clover silage content over a 6-day period.

On the 7–8 of January (6.3 hr of daylight), six of the eight ewes, in which all clinical signs of red clover effects had disappeared, were randomly assigned to two groups (3 each) which received one or two implants (a daily dose of 45 or 90 μg 17β-estradiol). The implants were removed after 14 days.

Five months after the initial treatment, the ewes were randomly redistributed to another two groups (4 each). Group I (mean body weight of 74 kg) was reexposed to red clover as previously described. Group II (mean body weight of 75 kg) was kept on hay and served as controls. All ewes were terminated at the end of this experiment and reproductive organs were collected, weighed, and examined for gross changes. In addition, blood samples were collected for subsequent progesterone and 17β-estradiol analysis. At the time of termination these animals were exposed to approximately 16 hr daylight.

Clinical Examinations. All ewes were clinically examined throughout the treatment to assess the physical condition of the reproductive organs. These examinations were carried out two or three times per week during the control periods, each exposure to red clover and 17β-estradiol. Udder growth and milky fluid secretion were noted, and the teat lengths and circumferences were measured. Averages for left and right teat measurements were recorded. Teat length was measured as previously described (13), except that permanent markers were used instead of tattoos. To measure teat circumference, we used thin, long nylon strings calibrated (mm) in the middle along a distance of 10 cm. The string was circled round the base of the teat, and the circumference was recorded. Contact between teat and string was ascertained without pressure on teat or space between teat and string.

The color of the vulva as well as signs of mucous discharge and edema were assessed. Arbitrary scores were assigned to changes in each parameter, ranging from 1 (no change) to 5 (severe change). The sum of the scores for changes in the three parameters was recorded for each period of vulva examination.

The uterus was examined by rectal ultrasonography using the Aloka scanner, model SSD-210DXII (Aloka Co. Ltd., Tokyo, Japan) with a real-time B-mode 2-dimensional scanner. It is equipped with a linear array 5 MHz transducer which is suitable for rectal use in nonpregnant, small ruminants (14). Measurements of uterine diameter were made with integral electronic callipers used for measuring linear distances. Because of errors in defining the endometrial limits we decided to remeasure the distances and standardize them on the ultrasonographs. The measurements from the pictures were from perimetrium (outer wall of uterine horn) to perimetrium.

Hormone Assays. Selected samples from each ewe were assayed for progesterone and 17β-estradiol to confirm the absence of ovarian activity.

Samples were assayed for progesterone by luminescence immunoassay (Amerlite; Kodak Clinical Diagnostics Ltd., Amersham, England) previously validated for canine plasma (15). Serial dilutions of ovine plasma containing high concentrations of progesterone produced curves parallel to the standard curve. The sensitivity of the assay system was <1 nmol/l. Intra-assay variation for progesterone was 13.5% and 6.0% for low and high assay controls, respectively. The corresponding interassay variation was 10.0% and 8.4%.

17β-Estradiol was analyzed (16) using standards supplied with the Coat-A-Count radioimmunoassay kit (Diagnostic Products Cooperation, Los Angeles, CA). Serial dilutions of ovine plasma containing high concentrations of 17β-estradiol produced displacement curves parallel to the standard curve. The detection limit of the assay was 5 pmol/l. The intra-assay variation calculated from the precision profiles of two assays was <17% for concentrations between 5.6 and 180 pmol/l. The interassay coefficient of variation for corresponding control samples was 7.6% (8.6 pmol/l) and 10.2% (198 pmol/l), respectively.

Statistical Analysis. Changes in teat length, teat circumference, and uterine diameter were expressed in percent of individual pretreatment values (i.e., 10 days before the beginning of red clover feeding).

Changes for all these parameters and vulva scores were computed and analyzed for treatment effects using the analysis of variance and the Duncan multiple range test from Statgraphics (STSC Inc., Rockville, MD). Probabilities <0.05 were considered statistically different.

Results

Feed Analysis. A homogeneous sample from the red clover silage, consisting of 24.6% dry matter, was analyzed to determine its nutritional quality and content of phytoestrogens. The red clover silage contained a total of 1.74 g estrogens (1.01 g formononetin, 0.03 g daidzein, 0.61 g biochanin-A, and 0.08 g genistein) per kg wet weight. Each ewe was fed 3.5 kg silage, corresponding to a daily intake of 6.1 g phytoestrogens (of which 3.5 g is formononetin). No estrogens were detected in samples of the hay or concentrates.

Clinical Findings. During the experiment in November, the teat length increased significantly on Day 4 in 100% of red clover fed ewes. The increase remained significant, and maximum length was reached on Day 15. Teat length was back to control levels on Day 39 (i.e., 25 days after withdrawal of the red clover) (Fig. 1). Teat circumference increased significantly on Day 1, peaked before withdrawal on Day 13 and dropped to control levels on Day 39 (i.e., 25 days after withdrawal of the red clover) (Fig. 2). Milky fluid secretion was observed in six of eight ewes. Two ewes began secretion on Day 5 and 10, respectively, and stopped on Day 25. The third showed secretion for only 9 days (from Day 19 to 28), and the fourth for 51 days (from Day 12 to 63). In the last two, starting on Day 7 and 13, respectively, milky fluid secretion lasted a few months. The amount of milky fluid varied from a few drops to a few milliliters per animal. In appearance, the secretion was initially clear, serous, and colored, but later resembled the milk. Mammary glands were noticeably more voluminous, and palpation revealed the presence of irregular, solid lumps of various sizes towards the end of the feeding period and long afterwards.

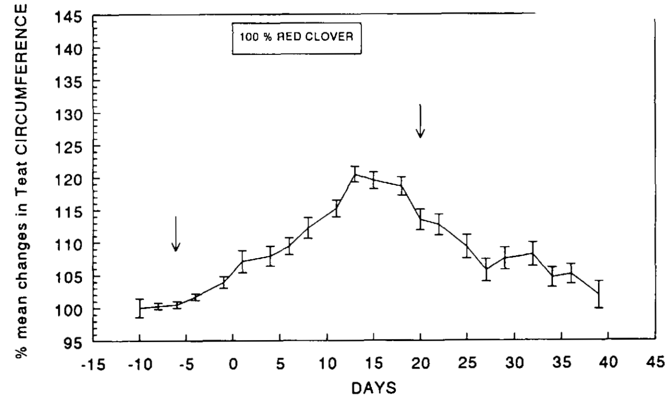

Figure 2. Mean (±SEM) changes (in percentage of initial values) in teat circumference (100% ± 3.9% is equal to 41.3 ± 1.6 mm) in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

The vulva gradually changed from pale to pink, and then to red with increasing edema. Vulva mucous discharges were negligible and brief in two ewes. On Day 4 in the ovariectomized ewes eating red clover diet, the vulva scores were significantly different from that seen before the initial treatment. Maximum changes were observed between Day 15 and 18, and the scores had dropped to control level on Day 36 (i.e., 22 days after withdrawal) (Fig. 3). The preliminary measurements made directly from the ultrasound images indicated that uterine diameter increased with exposure to phytoestrogens, and the standardized measurements from the photographs confirmed this trend

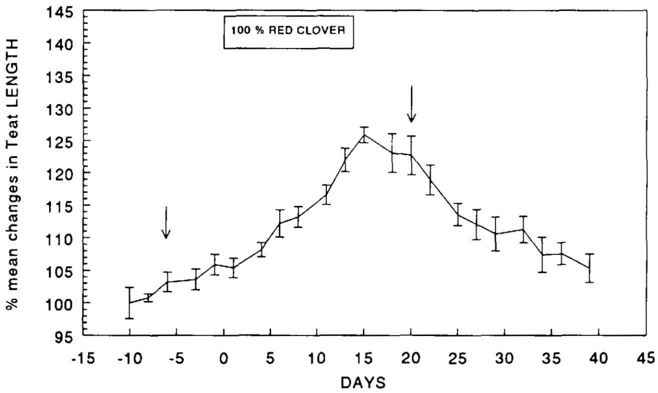

Figure 1. Mean (±SEM) changes (in percentage of initial values) in teat length (100% ± 6.8% is equal to 22.0 ± 1.5 mm) in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

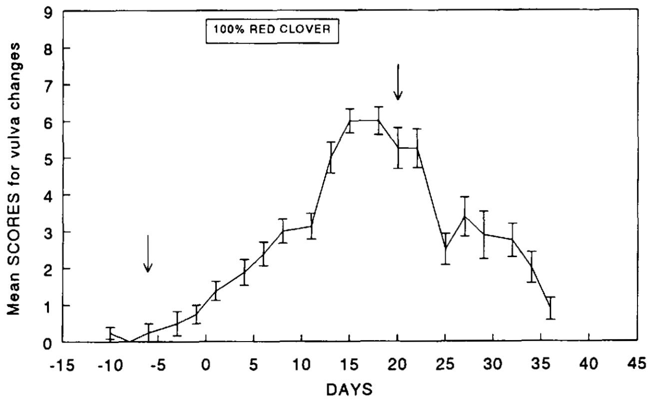

Figure 3. Mean (±SEM) vulva scores in ovariectomized ewes exposed to phytoestrogens in November. The timing of feeding with only red clover silage is shown by the box entitled "100% RED CLOVER." Arrows point to first and last day of feeding with red clover silage in addition to hay.

(data not shown). Uterine diameters tended to increase, with the highest value observed on Day 29 (i.e., 15 days after withdrawal of silage (124 ± 6%)) and subsequently decreased.

Plasma concentrations (mean ± SEM) of 17β-estradiol during the hay and red clover periods were 7.1 ± 0.7 pmol/l and 8.8 ± 0.9 pmol/l, respectively, and were not significantly different. Plasma concentrations for progesterone were below the detection limits (1 nmol/l) of the assay.

Abrupt changes occurred in all parameters of the reproductive organs observed in ewes treated with 17β-estradiol. There were already significant changes in teat length on Day 4 after insertion of one implant (Fig. 4). Two 17β-estradiol implants resulted in an increase of teat length on Day 12. The teats decreased in size to pretreatment values by Day 26 and 19 in ewes with one and two implants, respectively. In regards to teat circumference, the effects of 17β-estradiol were seen until Day 30–31 (i.e., 16–17 days after withdrawal of either one or two implants) (Fig. 5). Two 17β-estradiol implants resulted in a more abrupt and higher increase in teat circumference (Fig. 5) than that during exposure to phytoestrogens (Fig. 2). A milky secretion occurred in five of six ewes. Four ewes began to lactate on Day 9, and the fifth began on Day 14. The secretion lasted between 36 and 74 days.

In less than 24 hr, the vulva color changed abruptly from pale pink to red, with obvious edema. Only one ewe showed a mucoid vulva discharge, which lasted throughout the period with implants. The sum of the vulva scores was already significantly increased on Day 1 in ewes with one implant (Fig. 6). Thereafter, the scores remained significantly higher than those obtained from Day 0 until Day 20 (one implant) and Day 25 (two implants). Uterine diameters measured by means of ultrasonography tended to increase after insertion of the implants and tended to fluctuate around the same level as was seen during red clover feeding.

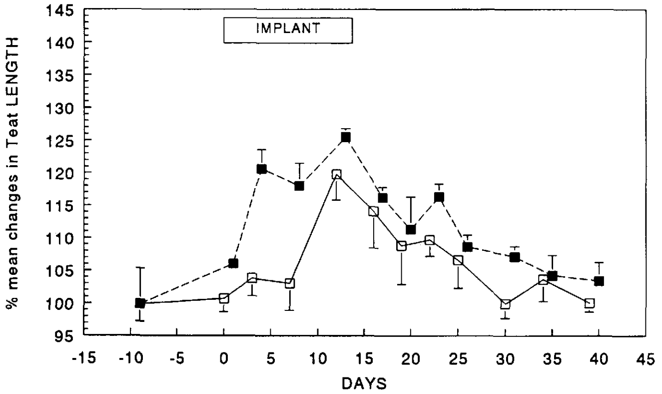

Figure 4. Mean (±SEM) changes (in percentage of initial values) in teat length in ovariectomized ewes treated with 17β-estradiol. Duration of treatment with one (■----■) or two (□—□) implants is shown by the box entitled "IMPLANT."

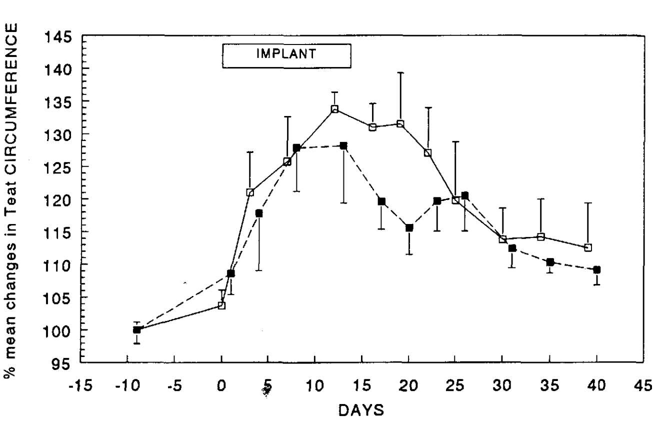

Figure 5. Mean (±SEM) changes (in percentage of initial values) in teat circumference in ovariectomized ewes treated with 17β-estradiol. Duration of treatment with one (■----■) or two (□—□) implants is shown by the box entitled "IMPLANT."

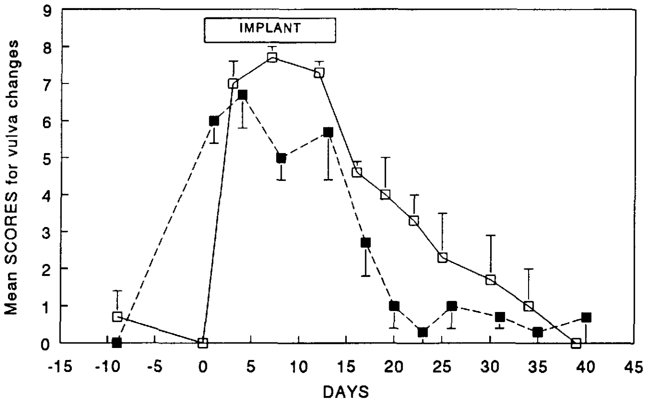

Figure 6. Mean (±SEM) vulva scores in ovariectomized ewes treated with 17β-estradiol. Duration of treatment with one (■----■) or two (□—□) implants is shown by the box entitled "IMPLANT."

The plasma concentrations (means ± SEM in pmol/l) of 17β-estradiol on Day 5–6, 7–8, and 12–13 after insertion of one or two implants were 43 ± 6, 36 ± 5, 32 ± 3 or 126 ± 40, 97 ± 38, 75 ± 23, respectively.

During the experiment in May, the percentage increase in teat length and circumference on Day 11 was significantly higher in ewes exposed to red clover than that in control animals (114% ± 4% vs 102% ± 2% and 113% ± 3% vs 101% ± 1%, respectively). On the same day, the sum of vulva scores significantly differed between experimental and control animals (7.0 ± 1.2 vs 0.3 ± 0.1). In general, there was no difference in responses to red clover exposure in November and May except for the sum of vulva scores. The changes in vulva were more dramatic in May, and on Day 1 and 11 the scores were 3.8 ± 0.6 and 7.0 ± 1.3, respectively, the values in both cases being significantly higher than those in November, as depicted in Figure 3. After slaughter in May, on Day 14, a significant difference was observed in the uterine weights between ewes fed silage and hay (65.3 ± 9.7 g vs 40.4 ± 2.8 g). Regarding the udder, only two of the four experimental animals showed an increase in weight (i.e., approximately 40% heavier udder than that in control ewes.

Discussion

This paper reports on the influence of red clover silage on the clinical status of reproductive organs in ovariectomized Swedish Finewool Landrace ewes.

The changes in teat length are in accordance with those previously reported in both ovariectomized ewes (17) and castrated rams (18) fed estrogenic clover. It was reported (19) that there was reasonable correlation between the mean increase in teat length at Day 14–17 and the mean uterine weight. The teat length method is simple and relatively sensitive, but the range of stilbestrol doses for which a linear log dose-response relationship was found to be narrow. The relationship between uterine weight responses and temporary infertility appears to be slightly better than that between teat length and infertility (20). Teat length responsiveness in wethers seemed to have been renewed by 35 days after their removal from estrogenically active clover pasture. In the present study, both teat length and circumference reached the pretreatment levels after 25 days from withdrawal of the silage. Mammary gland development and milky fluid secretion, which occurred in our ewes, have been reported in previous studies on sheep exposed to phytoestrogens (18, 21). However, the lobule-alveolar epithelial tissue pattern that normally develops prior to physiological lactation (22) is not established in lactating mammary glands from ewes grazing estrogenic pasture (18). In this experiment, the irregular solid lumps of tissue found during clinical examinations looked like developing tumors. Genistein, but not formononetin (23), and the estrogenic metabolite of formononetin called equol (24), can stimulate the growth of estrogen-dependent breast cancer cells *in vitro*. The estrogenic activity of biochanin-A and genistein in ruminants is limited to the first few days of exposure when the unadapted rumen microbes (2) cannot convert them to their nonestrogenic metabolites p-ethylphenol and phenolic acid (25). On the other hand, formononetin and daidzein in the rumen are mainly metabolized to equol (25), which is known to be estrogenic and relatively stable (8). The red clover used in this experiment contained relatively high concentrations of formononetin, which is thus considered to be the cause of the overall clinical changes observed in this experiment.

It was interesting to note the more dramatic increase of vulva scores in May than in November. However, the significance of that finding should be elucidated by using intact ewes. Swelling of the vulva was also reported earlier for ewes fed red clover (26), and it is a typical symptom of estrus in the ewe (27). The increased blood flow that accompanies hyperplastic and hypertrophic enlargement of the reproductive organs exposed to estrogens (2) is assumed to be responsible for the color changes observed in the vulva when ewes were fed red clover and treated with 17β-estradiol. It was reported (28) that ovariectomized ewes injected with estradiol benzoate for 12 days displayed estrus. One 17β-estradiol implant used in the present study created average concentrations similar to those previously reported (29, 30). These concentrations resemble the values of 17β-estradiol in ewes during the follicular phase of the estrous cycle (31).

In the present study, we attempted to use rectal ultrasonography to monitor changes in the uterus. The uterus became enlarged in ewes fed red clover and in ewes treated with 17β-estradiol, but decreased in size again once the red clover silage or estradiol implants were withdrawn. Unfortunately, we could not assess these uterine changes statistically owing to some uncertainties in the measurements. However, we assume that the validation of ultrasonography on several more animals may permit the studying of estrogenic effects in ewes by this noninvasive technique. The increase of uterus weight during feeding with red clover silage found here is in agreement with the previous studies (18, 19, 20). Additionally, it was reported (32) that 17β-estradiol treatment in ovariectomized ewes significantly increased uterine fresh and dry weights.

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