Infertility in Rabbits Induced by Feeding Ladino Clover.* (26129)

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It is now well established that potent estrogenic activity is extractable from a variety of commonly-used forage crops(1,2). Bennetts (3) describes a number of severe abnormalities of the reproductive tract of sheep pastured on Dwalganup strain of subterranean clover, and it is assumed that derangements of reproductive performance were caused by high estrogenic content of the clover (10 μg/100 g dry weight) because effects similar to those produced by injection of estradiol were duplicated by feeding the same strain of clover to guinea pigs(4). On the other hand, Kendall *et al.*(5) felt that partial infertility in New Zealand rabbits accompanied by hemorrhaging, numerous abortions and resorptions, and reduction in litter size, was due to a nutritional imbalance caused by feeding a diet of 50% soybean hay.

The research reported here was prompted by reports from several dairy farmers in New Hampshire of reproductive failure of heifers and dairy cows fed on a diet rich in Ladino clover. In each instance substitution of a timothy hay diet quickly corrected infertility. Interestingly, dairymen from other areas in the state, and in Mass. and N. J., indicated no reproductive problem on feeding Ladino. Nevertheless, occurrences of infertility seemed widespread enough to warrant at least pilot experimentation. Rabbits were selected as test animals with the thought that endocrine disturbances of reproductive processes induced by Ladino feeding, if any, could be worked out less expensively, in a shorter time, and in less space than if dairy animals were used.

Materials and methods. Domestic rabbits, principally of New Zealand and Flemish giant strains, 5-12 months old, were maintained in a well-ventilated room equipped with automatically-timed lighting from 7 a.m. to 9 p.m. throughout the year and on a diet of water and commercial rabbit pellets, *ad lib*. Fresh Ladino clover, in season, or Ladino hay (both grown on a University plot at Northwood, N. H.) was substituted for pellets in experimental animals. Extent of Ladino feeding for each test is given in Table I. Nutritive values of rabbit pellets and a diet of Ladino clover were virtually identical in important areas of crude protein, digestible protein, crude fat, ash, and N.F.E. content. Any animal showing signs of ill health was discarded and its data eliminated from final tallies. Three bucks of proven fertility were used for random matings, but never more than one mating was allowed per day. Both experimental and control does were permitted only one mating for any one experiment. Gestation periods were 30-34 days, and there were no known abortions. Hemorrhage, paralysis, or other signs of deficient nutrition were not observed. All does appeared to enter a span of anestrus extending from mid-November to early January; all experiments were consequently confined to the period 15 January to 15 November. Except for the short time mentioned, laboratory conditions induced, or at least did not prevent, continuous estrus in all does, so that they were available for experimentation at any time.

Results. Prefeeding and continuation of Ladino feeding during the supposed gestation period (Groups II, III, Table I) resulted in total infertility. When these animals were returned to a pellet diet 35 days after original mating (56 days after start of Ladino diet) all refused to mate and required 3-36 (avg. 24) days before estrus reappeared. In contrast it should be noted that does mated readily after 3 weeks of Ladino prefeeding earlier in the same experiments. If Ladino feeding was restricted to either prefeeding or

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TABLE I. Effect of Ladino Feeding on Reproductive Performance of Female Rabbits.

Group	No. of animals	Diet prior to mating	Diet from day of mating	No. animals littering	Avg No. in litter
I (control)	20	Pellets	Pellets	18	8.1
II	6	Ladino clover (3 wk)	Ladino clover	0	0
III	"	Ladino hay (2½ wk)	Ladino hay	"	"
IV	"	Pellets	" "	2	5.5
V	"	"	Pellets for 96 hr, then Ladino hay	3	7.7
VI	"	Ladino hay (2½ wk)	Pellets	3	7.0
VII	5	*Idem*	Ladino hay for 96 hr, then pellets	3	8.3
VIII	4	"	Ladino hay	—	0 *
IX	"	Pellets	Pellets	(4)	7.2*

* Implantation sites or embryos in laparotomized animals.

a part or all of the gestation period, fertility was not so seriously affected (groups IV-VII), but on the basis of control performance there was definite impairment of reproduction. All experimental animals which failed to litter were subsequently shown to be normally fertile after return to a pellet diet and a short period of rest. One animal in each of groups VIII and IX was sacrificed at 5, 10, 15, and 20 days following mating and exploratory laparotomies performed. No blood points, implantation sites, embryos, or abnormalities of reproductive tract were found in any of the Ladino-fed animals (VIII), and only one corpus luteum was discovered (on one ovary of the 15-day animal). All pellet-fed rabbits (IX) appeared to be carrying out gestation normally.

Ladino clover and hay were assayed using a 22-day-old rat test, measuring increase in uterine weight 48 hours after the start of twice-daily injections. Samples contained between 25 and 35 μg of estrogenic activity (expressed as diethylstilbestrol) per 100 g oven-dried forage. Rabbit pellets did not contain measurable estrogenic activity. Average daily consumption of pellets or clover per animal was approximately 190 g oven-dried weight. This means that rabbits on Ladino may have received as much as 55-65 μg per day of diethylstilbestrol equivalent orally.

Discussion. There is at present no obvious explanation for Ladino's action on rabbits. Ladino appeared to have an even greater estrogenic content than Dwalganup subterranean clover tested by Curnow *et al.*(4), but use of different bioassays may mean that estrogenic levels reported are not wholly comparable. When Ladino was fed (about 90 g daily) to newly-weaned rabbits for 2-4 weeks there was no premature stimulation of the reproductive tract over that of pellet-fed littermate controls. These young rabbits ingested circa 30 μg daily of diethylstilbestrol equivalent as contained in Ladino, yet they were unaffected, indicating that in spite of the high estrogen level of Ladino as ascertained by injection assay in rats, physiologically inactive amounts were apparently absorbed when given rabbits orally. The question is immediately raised whether the depressing action of Ladino on fertility of rabbits may be entirely unrelated to its estrogenic content.

Laparotomies of Ladino-fed (group VIII) animals indicated that ovulation had not occurred. Failure of ovulation could be an explanation for infertility in those animals pretreated with Ladino before mating but probably not for those which had no Ladino previous to mating, unless the effect of Ladino is so rapid as to exert some influence in the 10-hour interval elapsing between mating and supposed ovulation time (as in group IV). Results of all experiments could be explained on the basis that Ladino interfered with either ovulation or implantation. It is interesting that Ladino silage, obtained from the same Northwood plot, did not adversely affect conception rate of an experimental group of Holstein heifers.

Summary. Feeding Ladino to female rab-

bits impaired reproductive performance, infertility being complete when clover was fed both prior to and during gestation periods. Cause is unknown but failure of ovulation and implantation was observed, and evidence indicates that infertility may be unrelated to estrogenic content of the clover.

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Studies on Binding of Tetracyclines by Dog and Human Plasma.* (26130)

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Several investigators have studied the binding of tetracyclines by dog and human plasma. Binding of chlortetracycline in man has been reported by Sirota and Saltzman(1), Kunin *et al.*(2) and Kivman(3). Kunin *et al.*(2) have studied binding of demethylchlortetracycline in man, and Kohn *et al.*(4) in dog. The extent of protein binding of tetracycline by human plasma was investigated by Kunin *et al.*(2) and Kivman(3), and in dog plasma by Pindell *et al.*(5) and Kohn *et al.*(4). Not all the investigators agreed in their findings and different values were reported for one specific antibiotic and species.

The present paper deals with a comparative study of binding of demethylchlortetracycline with chlortetracycline and tetracycline by dog and human plasma. Extent of protein binding was determined by the dialysis equilibrium method, at drug concentrations approaching therapeutic levels and under physiological conditions of temperature and pH. The effect of temperature and pH on binding of tetracycline was also studied.

Method. The experiments were performed with a pool of dog plasma† (5 males and 6 females) and a pool of human plasma† (4 healthy males). Concentration of protein in plasma was determined by micro-Kjeldahl analyses. Distribution of plasma proteins was determined by free electrophoresis in the Spinco Model H electrophoresis apparatus.‡ Tetracyclines used were radioisotopically labeled as follows: 7-tritio-tetracycline hydrochloride, C^{14} demethylchlortetracycline hydrochloride and C^{14} chlortetracycline hydrochloride. Corresponding specific radioactivities were 13, 0.43 and 0.18 μc/mg. The purity of these tetracyclines, checked by various procedures including microbiological assay, spectrophotometric assay and paper chromatography, was 90% for demethylchlortetracycline hydrochloride, 95% for chlortetracycline hydrochloride and 100% for tetracycline hydrochloride. The technic followed for dialysis was developed by Roepke *et al.*(6). Small volumes (0.005-0.040 ml) of aqueous solutions of the tetracyclines were added to the plasma or to the buffer from a micro syringe-burette.§

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† Obtained from heparinized blood centrifuged with a layer of oil.

‡ Determined by Mr. M. C. Davies, Biochem. Research Dept.

§ Manufactured by California Laboratory Equipment Co.