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Canine estrus suppression with mibolerone delivered by an intravaginal device

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CANINE ESTRUS SUPPRESSION WITH MIBOLERONE DELIVERED BY AN INTRAVAGINAL DEVICE

Iowa State University PH.D. 1981

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Canine estrus suppression with mibolerone delivered by an intravaginal device

by

Keith Edward Magoon

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INTRODUCTION

The number of unwanted stray and abandoned dogs is increasing in the United States, and some of these free-roaming animals cause property damage, attack other animals and man and spread diseases. Control of reproduction is an acceptable solution to the problems they create, and the few methods presently available for successful reproduction control in the canine have limited application due to cost, inconvenience and owner apathy. The most direct method of population control is to sterilize the female or inhibit her sexual response. Presently, the methods utilized in pregnancy prevention of the bitch are ovariohysterectomy, a mechanical device or confinement to prevent mating and the oral contraceptive drugs (megestrol acetate and mibolerone). Less costly and equally effective control methods need to be developed.

To the knowledge of the author, there have been no reports on the use of intravaginal or intrauterine drug delivery in the canine to control estrus. Intravaginal and intrauterine administration of estrus controlling agents has had limited success in a number of other animal species (Doyle, 1975; Casas and Chang, 1970; Faulkner and Hopwood, 1967; Robinson, 1965; Roche, 1976; Sreenan and Mulvehill, 1975).

The aim of this project was to develop an intravaginal delivery of a contraceptive drug for the control of canine reproduction. To be successful, the method developed had to meet the following criteria:

1) A drug delivery device had to be designed to deliver a suitable contraceptive drug to the animal.

2) The delivery device had to be retainable within the canine vagina and cause no gross side effects.

3) The contraceptive drug chosen for delivery had to be capable of diffusing across the vaginal mucosa and into the blood stream, without causing adverse side effects.

4) The end effect had to result in estrus suppression.

LITERATURE REVIEW

Estrous Cycle in the Bitch

The estrous cycle (reproductive cycle) is initiated in the bitch at puberty. In the female Beagle, the first estrous cycle begins to function between 9 and 14 months of age (Andersen, 1970). The phases of the estrous cycle are proestrus, estrus, metestrus, diestrus, anestrus and sometimes pregnancy. The average duration for a complete cycle, in the Beagle, is 220 $\pm$ 56 days (Andersen, 1970) with a 1 month variation considered within the standard error of the mean cycle length (Sokolowski, Stover and Van Ravenswaay, 1970; Jöchle and Andersen, 1977).

The first signs of proestrus are usually a serosanguineous vaginal discharge accompanied by swelling of the vulva. The erythrocytes contained in the vaginal discharge are derived from the rapid proliferation of the endometrium by the process of diapedesis. Male attraction begins due to the female emission of pheromones, but the female does not permit coitus during this phase of the cycle. Proestrus may last 7 to 14 days (Andersen, 1970).

Microscopic examination of appropriately stained vaginal smears may also detect the onset of proestrus. In these preparations, increasing cornification of the vaginal epithelial cells will be seen (Roszel, 1975; Gier, 1960). In

early proestrus, there are many erythrocytes along with superficial epithelial cells. Neutrophils and cells of deeper epithelial layers, parabasal and intermediate cells are also present during early proestrus. It has been noted that in mid-proestrus, the superficial cells have frayed cytoplasmic borders and the neutrophils are generally few in number.

During the transition from late proestrus to early estrus, a vaginal smear with erythrocytes, superficial cells and occasionally anucleated squamate cells, is seen. The superficial cells are less likely to have frayed edges and neutrophils are absent. In early estrus, the vulva is swollen but less turgid and the vaginal discharge is scanty and less red than during the proestrus phase.

Estrus is the period of sexual receptivity in which the female will assume a breeding stance and allow coitus to occur. The length of this phase of the reproductive cycle has been reported to range from 7 to 10 days (Andersen, 1970). The smear pattern at this time represents a highly proliferative vaginal epithelium in response to estrogen. Late estrus brings the reappearance of intermediate cells, superficial cells and a variable number of erythrocytes in the vaginal smear (Roszel, 1975).

Opinion is divided on what occurs after estrus, with some researchers characterizing the subsequent period as metestrus and others, diestrus. The subject is further

complicated by the fact that metestrus and diestrus are defined in different ways (Sokolowski, 1977; Holst and Phemister, 1974; McDonald, 1976; Jöchle and Andersen, 1977; Andersen, 1970). For a possible working definition, metestrus can be considered as the period of corpora lutea development and diestrus as the period of luteal hormonal activity. Regardless of how the period is characterized, it stands that sexual receptivity ceases as estrus ends and metestrus begins. There is a rapid decrease in vulvular swelling and vaginal discharge. Early in metestrus, the vaginal smears have neutrophils mixed with parabasal cells, intermediate cells and a few superficial cells (Roszel, 1975). Further into metestrus the superficial cells disappear and increased numbers of intermediate and parabasal cells along with some neutrophils are seen. Smears taken during metestrus may contain foam cells or metestrum cells (Roszel, 1975) and considerable debris (Gier, 1960).

Anestrus follows the cessation of luteal activity and is the phase of ovarian inactivity or sexual quiescence. Smears taken during this period may vary as to the predominant cell type. Neutrophils may be sparse or numerous, depending upon the condition of the vagina. Intermediate cells and parabasal cells are present and generally superficial cells are absent (Roszel, 1975).

The reproductive cycle is controlled by a complex

interrelationship of hormonal activity with ovarian, uterine and vaginal reactions. A gradual rise in follicle stimulating hormone (FSH) in the blood plasma begins a few weeks before the onset of proestrus. Gonadotropin-releasing hormone (GnRH) is responsible for the release of pituitary FSH into the blood, but the mechanism initiating GnRH is unknown (Sokolowski, 1977). FSH stimulates ovarian activity and results in follicular development.

Follicles approach maturity during proestrus and as they develop to ovulatory size, they secrete and release estrogens into the blood. Estrogen stimulates the vaginal epithelium causing cornification of the cells and thickening of the vaginal mucous membranes.

As a result of estrogen stimulation there is a continuation in the increase in uterine size due to the proliferation of the uterine epithelium and increased blood flow. The peak of blood concentration of estrogen correlates with the beginning of sexual receptivity by the female (Reimers et al., 1978; Nett et al., 1975)(see Figure A1, Appendix A). There is some disagreement about the level of estrogen (estradiol-17B) attained during late proestrus. Levels of 14.0 $\pm$ 2.4 pg/ml (Reimers et al., 1978) to 56.7 $\pm$ 6.5 pg/ml (Nett et al., 1975) have been reported. A possible explanation for the differences may be due to lipid concentration variations in the blood due to feeding, which affect the

radioimmunoassay of estrogen (Reimers et al., 1978). The significant point is that estrogen levels reach a peak in late proestrus. Present evidence indicates that ovulation occurs in partial response to the high plasma estrogen levels (Bell, Christie and Youngglai, 1971). The estrogen levels decline rapidly after peaking and reach a baseline level by day 7 of estrus.

Most researchers are of the opinion that rising estrogen levels during proestrus stimulate the release of luteinizing hormone releasing hormone (GnRH) and that the GnRH then initiates the release of luteinizing hormone (LH) from the pituitary. The effect of LH is to stimulate the developing follicle towards maturation, estrogen production and ovulation. FSH and LH are both thought to have a role in ovulation, but ovulation will not occur without an increase in LH in the blood. Ovulation occurs within 24-48 hours after estrus begins (Sokolowski, 1977; Phemister et al., 1973; Holst and Phemister, 1975). Blood levels of both FSH (56.3 $\pm$ 8.7 ng/ml) and LH (1.9 $\pm$ 1.1 ng/ml) are low during proestrus and coincidentally increase to a maximum (FSH to 167.6 $\pm$ 36.9 ng/ml and LH to 31.1 $\pm$ 4.9 ng/ml) on the first day of estrus (Reimers et al., 1978). LH rapidly declines to less than 2 ng/ml by the second day after the onset of estrus and remains at that level until the next proestrus (Reimers et al., 1978; Smith and

McDonald, 1974). FSH declines slowly to 69.2 ± 14.7 ng/ml by day 6 of estrus, but does not remain at this lower level throughout diestrus (Reimers et al., 1978). Levels rise again by days 28-30 after ovulation and are reported to remain high through days 55-58 but no explanation is given for these high levels of FSH (Reimers et al., 1978). FSH levels then decline to a baseline level and remain until a few weeks preceding the next proestrus.

Following ovulation, the follicle now without the ovum, becomes the corpus hemorrhagicum, which quickly develops into the corpus luteum. The function of the corpus luteum is to produce progesterone. Increasing progesterone levels act with the declining estrogen levels during estrus to promote positive sexual behavior in the female. This behavior consists of standing firmly, deviating the tail and displaying the vulva. Progesterone stimulates the uterine endometrium and myometrium to undergo hypertrophy, hyperplasia and marked glandular development.

Blood levels of progesterone are lowest during proestrus (2.0 ± 0.3 ng/ml) and have been found to increase gradually to approximately 22.2 ± 4.5 ng/ml by the sixth day after estrus begins (Reimers et al., 1978). Whether or not the animal becomes pregnant, the progesterone levels remain elevated for 28-30 days following the onset of estrus

(Reimers et al., 1978; Concannon et al., 1977; Nett et al., 1975; Smith and McDonald, 1974). A gradual decline in progesterone levels then ensues and by day 55-58 the level has dropped to 3.4 $\pm$ 0.7 ng/ml (Reimers et al., 1978; Concannon et al., 1977; Smith and McDonald, 1974).

Venoarterial Pathways

In recent years, some attention has been given to vaginal, uterine and ovarian vasculature, especially uterine and ovarian vasculature. A concept involving internal regulation of physiological processes through local venoarterial pathways has evolved. The basis for this concept is the fact that an organ or a compartment of an organ can have a regulatory influence on another organ or compartment that is located nearby. The regulatory influence is accomplished through a local pathway involving the veins of one organ and the arteries of the other (Ginther, 1974; Ginther, 1976). It has been proposed that a uterine luteolysin is transported through a venoarterial pathway, which involves the veins draining the uterus and the ovarian artery. The luteolysin causes regression of the corpus luteum. The nongravid uterus produces the luteolysin, which passes into the uterine veins and venules. There is extensive contact between these veins and venules and the wall of the ovarian artery in animals such as the sheep

(Del Campo and Ginther, 1973; Mapletoft and Ginther, 1975). In the areas of contact the luteolysin diffuses from the veins and venules into the ovarian artery, where it is transported to the ovary to act upon the corpus luteum. Diffusion could be aided by the fact that the wall of the artery and vein is thinner in the area of apposition of the vessels, at least in sheep and cattle (Ginther, 1974).

Due to the close anatomical arrangement of arteries and veins near the ovary, a venoarterial pathway may exist in the bitch (Del Campo and Ginther, 1974). However, such a pathway does not seem functional in the canine, because once the corpus luteum is formed, progesterone is secreted essentially at the same levels for 60-70 days whether there is a pregnancy or not. Other local venoarterial pathways cannot be ruled out in the canine and one of these could possibly serve to take up a steroid from the uterus and/or vagina, which would in turn effect ovarian activity.

Surgical Prevention of Fertility

A very positive method of control of reproduction is ovariohysterectomy. It not only provides a control of reproduction, but eliminates the problems associated with estrus and the common intrauterine diseases including mucometra and pyometra (Djerassi et al., 1973; Wildt and

Seager, 1977). Even though ovariohysterectomy is effective in making the pet sterile it has met with the following oppositions: 1) Some owners do not want their pet irreversibly sterile, and 2) some owners believe that the cost of an ovariohysterectomy is prohibitive (Djerassi et al., 1973; Wildt, Kinney and Seager, 1977). The expense of surgery is the most significant reason that ovariohysterectomy has not been utilized more fully for fertility control. Some cities are using public spay programs, reducing normal costs, but it is generally agreed that the program is only a partial solution to the overpopulation problem (Burns, 1972; Faulkner, 1971; Hummer, 1975).

Methods of performing tubal ligations with metallic clips or electrocautery transection of the oviducts has been under investigation (Wildt and Seager, 1977). Although this method may prevent fertilization in the female, it would not prevent estrus and hence the attraction of males, nor does it reduce the probability of future uterine problems developing. In addition, expensive laparoscopy equipment ($3,000 estimated cost in 1977), is required to perform the tubal ligation.

Intravaginal Devices for Fertility Control

An intravaginal device, Agrophysics Breeding Control Device (ABCD) is presently available from Agrophysics, Inc., San Francisco, California (Hauge, 1974). The device is designed to prevent penile insertion into the female and "coital-lock". Vaginal fluids are reported to drain posteriorly around the smooth surface of the device with no interference regarding normal discharges or urination (Hauge, 1974). In addition, no adverse histopathological reactions or behavior changes are reported to occur while the device is intact. Estrus, vaginal discharge and attraction of males still occur while the device is in place. Retention of the vaginal device is dependent upon proper fitting and insertion at the appropriate phase of the cycle. However, mating will occasionally occur even with the device properly positioned. To resolve the problem, the manufacturer has been investigating a modified ABCD fitted with a copper ring (Wildt, Kinney and Seager, 1977). It is hoped that ionized copper will cause the vaginal secretions to be spermicidal.

Pharmacological Control of Fertility

A number of oral and injectable contraceptive drugs have been studied and found to control fertility, but they often cause pathological changes in the uterus making an ovariohysterectomy necessary. All known exogenous progestogens can cause cystic endometrial hyperplasia, endometritis and mucometra (Wildt and Seager, 1977). The following progestogens have been studied: Progesterone (Bell and Christie, 1971; Capel-Edwards et al., 1973; Christie and Bell, 1970; Sokolowski, 1974; Cox, 1970); Medroxyprogesterone Acetate (MAP) (Bell and Christie, 1971; Anderson et al., 1965; Brodey and Fidler, 1966; Bryan, 1973); 17α-acetoxyprogesterone (Bell and Christie, 1971; Bryan, 1960); Norethisterone Acetate (Cox, 1970; Halnan, 1965; Prole, 1974a; Prole, 1974b); Delmadinone Acetate (Stabenfeldt, 1974); Melengestrol Acetate (Zimbelman et al., 1970); Chlormadinone (Nelson et al., 1972); Megestrol Acetate (Ovaban) (Bell and Christie, 1971; Sokolowski, 1974; Cox, 1970; Schering Corp., 1975); and Proligestone (Van Os and Oldenkamp, 1978).

Of these progestogens, only megestrol acetate (Ovaban) used for the postponement or prevention of estrus in the canine has been approved for commercial use in the United States. Megestrol acetate is administered orally. The

mechanism of action is unknown but some believe it acts by inhibiting the production and release of hormones and/or local prevention of ovarian follicle growth, estrogen secretion and ovulation (Schering Corp., 1975). Megestrol acetate has been approved for a maximum of 32 days continuous use and should not be repeated for more than two consecutive estrus cycles. Excessive use of the drug may cause cystic endometrial hyperplasia (Van Os and Oldenkamp, 1978).

Estrus inhibition with minimal side effects occurs with subcutaneous androgen implants, such as testosterone (Simmons and Hamner, 1973). Anestrus was maintained for up to 840 days, in 60 females studied. The disadvantages of the testosterone implants include the possible need for more than one implant, depending upon the weight of the animal, and the need for general anesthesia to permit surgical implantation in the subcutaneous tissue of the flank. In addition, there may be some masculinization including enlargement of the clitoris and increased vaginal discharge during the implantation period, but these effects are considered minimal. The precise mechanism of action of testosterone is unknown, but it is thought to suppress the hypothalamic-pituitary gonadotrophin release system (Simmons and Hamner, 1973).

Mibolerone (17β-hydroxy-7α, 17-dimethylestr-4-en-3-one) an androgenic anabolic steroid has been used orally as a

treatment for preventing estrus and found to have minimal adverse side effects (Sokolowski, 1974). The drug is marketed for oral administration by the Upjohn Company (Kalamazoo, Michigan) under the trade name Cheque.R Estrus is inhibited only for the duration of a daily dosage, which, to be effective, must be initiated prior to the beginning of proestrus. The daily dose level is calculated according to the body weight of the bitch, with some breed variations (Sokolowski, 1976, 1978a).

Primary and secondary follicular development on the ovary still proceeds when mibolerone is given, but the follicles do not mature to ovulatory size. The LH surge is blocked by mibolerone through an unknown mechanism (Upjohn Company, 1979; Reimers, 1978; Sokolowski, 1976). Thus, estrus, ovulation and corpus luteum formation are prevented during the administration of mibolerone (Sokolowski, 1976; Reimers, 1978).

Mibolerone is rapidly metabolized and the breakdown products are excreted in the urine and feces. The highest tissue concentrations of mibolerone have been found in the liver, anal glands and reproductive organs (Sokolowski, 1976).

The biological effects of mibolerone have not been thought to be detrimental to the animal's health either during or after treatment (Sokolowski, 1976, 1978b; Sokolowski

and Geng, 1977). Beagles have been subjected to 30 µg/day, 90 µg/day and 200 µg/day drug dose levels for up to 730 days. The normal dose of mibolerone to control estrus in the beagle was 30 µg/day, but treatment levels of 200 µg/day for 730 days were not harmful (Sokolowski and Geng, 1977). Vaginal irritation and clitoral enlargement were more prominent in the immature than in mature females treated with 200 µg/day for 730 days (Sokolowski, 1978b). A vaginal discharge consisting of a white viscid liquid containing leucocytes has been associated with vaginal irritation and clitoral enlargement. Periodic episodes of excessive lacrimation also occurred in some test animals. Evaluations including clinical chemistry, hematology, urinalysis, gross pathology and histopathology have shown no drug related toxic effects. The only drug related effects in the female have occurred when daily oral administration has been at 3,000 µg and above (Sokolowski, 1976, 1978b). These high mibolerone treatment levels have produced a reduction in stainable lipid in the adrenal cortices, a thickening of the myometrium and endometrium and enlargement of the clitoris.

Mibolerone, administered orally, is presently approved for a two year period of continuous use in the female canine for the control of estrus.

Pharmacological Inducement of Asexuality

Sterility has been induced in young female mice and rats by single injections of testosterone propionate (Barraclough, 1961).

Female neonate puppies have been injected with testosterone propionate, depo-testosterone cyclopentylpropionate, or medroxyprogesterone acetate to inhibit sexual maturation and induce sterility. These androgen and progestin derivatives were ineffective in inducing sterility or delaying age of first estrus in the canine because hypothalamic differentiation occurs before birth in this animal (Zimbelman and Lauderdale, 1973).

Immunological Control of Canine Fertility

Immunological methods for neutralizing endogenous hormone have met with limited success as a means for suppressing the reproductive function. Female beagles immunized with human chorionic gonadotropin (HCG) developed antibodies to the HCG. Unfortunately, the antibodies were not cross-reactive with the beagle pituitary gonadotrophins and reproductive impairment was not produced (Al-Kafawi et al., 1974). Immunization of female beagles with bovine LH or ovine gonadotropins has stimulated an immunological

response producing a temporary reproductive failure, but additional injections of bovine LH or ovine gonadotropins have had decreased effectiveness (Faulkner et al., 1975).

Irradiation for Fertility Control

Single and multiple irradiation (x-ray treatment) applied directly on surgically exposed ovaries has produced infertility, which in some cases was reversible (Lee and Carlson, 1965, 1967). Whole-body x-ray treatment during early puppyhood (birth to two months of age) has provided some success in causing abnormal follicles in the ovaries but not so in older dogs (Andersen and Simpson, 1970). Multiple x-ray treatments are necessary to produce infertility and cannot be considered practical or economical as a fertility control method in dogs.

Implantable Drug Delivery Systems

The concept of implantable drug delivery systems has been reported in use as early as 1937, when R. Deansley and A. S. Parkes subcutaneously implanted compressed pure crystalline estrone in chickens (capons) (Blackshear, 1979).

Silicone-rubber capsules are the simplest of the delivery systems. They are composed of a silicone rubber capsule which serves as a diffusible membrane for the drug

contained within it. Drugs of low molecular weight and many hormonal preparations diffuse readily through the silicone (Kincl et al., 1968; Dziuk and Cook, 1966; Doyle, 1975). The silicone-rubber capsules have been found to produce little to no inflammation of body tissue and are considered very acceptable for implantation.

An implantable infusion pump (trade name Infusaid) is presently being manufactured and is considered by some people to be an improvement on devices that deliver through membranes (Blackshear, 1979). The Infusaid consists of a cylindrical disk about the size of an ice-hockey puck. The cylindrical disk is divided inside by a cylindrical bellows, forming an inner and outer chamber. Medication is contained in the inner chamber and a fluorocarbon in a liquid-vapor combination is in the outer chamber. The operation of the pump is based on the concept that a liquid in equilibrium with its vapor phase will exert a constant vapor pressure at a given temperature, regardless of volume. Pressure created in the outer chamber pushes against the bellows, which then forces the medication through a delivery cannula connecting the inner chamber and a vein. The cylindrical disk including bellows is made of titanium, which is impermeable, light in weight and compatible with body tissues.

An osmotic dispenser, patented in 1975 by T. Higuchi,

has been developed by the Alza Corporation, Palo Alto, California, into the now commercially available Alzet™ Osmotic Minipump. The Higuchi-Alza Minipump consists of an expandable solution-containing chamber which has an osmotic membrane exposed to body fluids, a drug reservoir, and a flow control "capillary" tube to permit the slow discharge of the drug as the expandable chamber increases in volume due to osmotic pressure (Higuchi, 1975; Yates, 1977). The Minipump has been used successfully as a subcutaneous implant infusing several different drugs, individually, over a four day period (Bowers and Folkers, 1976).

A more complex implantable capsule for delivering drugs has the unique feature of a magnetic "switch" (Merrill, 1972). Only a portion of the capsule wall is constructed of a drug-permeable material with the remainder of the capsule wall nonpermeable to the drug. Utilizing an external magnetic force, an inner nonpermeable, magnetically-moveable wall is activated to expose the drug to the permeable membrane allowing diffusion into the permeable membrane and subsequently into the body. Drug diffusion is stopped by reversing the external magnetic force, which moves the inner nonpermeable wall between the drug and the permeable membrane.

For implantation, a ballistically implantable projectile has been adapted to carry drugs into an animal and thereafter

provide sustained release of the drug (Drake and Paul, 1976). The bullet shaped ballistic projectile is designed to contain a drug within a cylindrical cavity in the basilar portion of the projectile. The walls of the projectile may be composed of a microporous, insoluble material, such as, polysiloxane or a soluble material, such as, gelatin or hydroxypropyl cellulose in order to provide a sustained release of the drug within the animal.

An intravaginal device for dispensing drugs to human females has been designed (Tillmann, 1970). The device is a hollow rod having a rounded cap with small apertures at one end through which the drug is delivered. The drug is forced through the small apertures by pressure from a dosing piston which has some similarity to the plunger found in a syringe. The stem of this dosing piston is designed as a feed gear which can be activated to deliver a specific amount of drug. Finger or thumb pressure on the base end of the device causes the feed gear to push forward and hence dispense the desired amount of drug. This device remains intravaginal only during the time required for each drug dispensation.

Basic Steroid Diffusion Through Silicone-Rubber

A great quantity of research on contraceptive drugs has established the fact that reproductive steroid hormones do pass through silicone-rubber (Kincl et al., 1968; Dziuk and Cook, 1966; Doyle, 1975; Tatum, 1970). Transmission of a steroid through a silicone-rubber membrane free from flaws occurs by an activated diffusion process (Dennis and Larson, 1977). The steroid first dissolves in the surface layers, followed by migration through the silicone-rubber membrane under a concentration gradient, and then is detached from the low concentration surface. The diffusion mechanism involves a concept of segmental polymer motion to form a free volume in the membrane; the available free volume is then occupied by a steroid molecule, which then migrates across and detaches from the opposite side.

Permeability is a function of both the properties of the diffusing molecule and of the silicone-rubber. Molecular size, shape, polarity and solubility parameters of the diffusing molecule greatly affect their transmission rate (Dennis and Larson, 1977). Crystallization, degree of cross-linking and rigidity of the silicone-rubber backbone also influence diffusion (Dennis and Larson, 1977). Other factors having considerable effect on diffusion are: 1) concentration of the diffusing molecules, 2) temperature, 3) membrane

thickness, and 4) membrane surface area exposed to the diffusing molecules.

Steroid diffusion across a silicone-rubber membrane has been found to be proportional to the thickness and area of the membrane (Kincl et al., 1968). Increasing the area exposed to the steroid results in a proportionate increase in diffusion. Increasing the thickness of the membrane causes a proportionate decrease in diffusion. The diffusion rate (at standard temperature and pressure) has been expressed as μg of steroid/time period/membrane area exposed/membrane thickness (Kincl et al., 1968).

Temperature effect on the rate of diffusion is dependent on the nature of the material diffused. If the diffusing material consists of relatively small and nonpolar molecules, the permeability of silicone-rubber decreases with increasing temperature due to decreasing solubility (Dennis and Larson, 1977). Permeability increases directly with increasing temperature when the diffusing molecules undergo certain conditions of high energy of activation for diffusion (Dennis and Larson, 1977).

Intravaginal and Intrauterine Delivery of Drugs for Estrus Control

To the knowledge of the author, intravaginal or intrauterine drug delivery systems have not been used for canine estrus control. Estrus controlling drugs delivered by the intravaginal or intrauterine route have had limited success in a number of other animal species.

Vaginally inserted polyurethane sponges impregnated with a progestogen have successfully controlled the onset of estrus and ovulation in the cyclic ewe (Robinson, 1965). Estrus and ovulation were blocked while the impregnated sponges were intravaginal. Upon removal of the sponges, estrus and ovulation began 2-3 days later in the mature ewe. The sponges produced vaginitis but this had no effect on fertility.

Intravaginal suppositories impregnated with 9α-fluoro-γ-11β-hydroxy-17α-acetoxyprogesterone,1, 2-^{3}H have been inserted into a number of ewes and drug uptake monitored (Faulkner and Hopwood, 1967). Between 12 and 24 percent of the drug was found to be absorbed from the suppository.

Progestogen-impregnated vaginal suppositories inserted for 20 days have been used to synchronize estrus in cattle. Suppository retention has been low and subnormal fertility rates have been reported for the first estrus following the treatment period (Sreenan, 1974, 1975). A follow-up study

(Sreenan and Mulvehill, 1975) compared the suppository treatment for 10 and 20 days along with an intramuscular injection of progesterone and estradiol benzoate at the time of insertion. Suppository retention was above 93 percent during the 10-day period, but decreased to 86 percent over the 20-day period. Fertility at first estrus was found to be better after the 10-day intravaginal treatment than after the 20-day intravaginal treatment.

A silastic coil impregnated with progesterone has been placed intravaginally for 12 continuous days in the bovine. High coil retention, controlled estrus and normal fertility were the results of the 12-day treatment (Roche, 1976).

In rats (Doyle, 1975), monkeys and humans (Scommegna et al., 1970), intrauterine delivery of progestins has been investigated mainly for their effect in decreasing uterine motility and thus improving the retention of existing intrauterine contraceptive devices (IUD's). In the rat, the primary aim was to establish which compounds might have potential for human use in retaining IUD's. All the progestins tested were effective in enhancing retention of the silicone-rubber IUD's with melengestrol acetate being most effective.

Small doses of progestins have been shown to be contraceptive without suppressing ovulation (Scommegna et al.,

1970). The normal menstrual patterns of primates were not significantly altered, even though endometrial changes did take place, therefore, it has been suggested that implantation may be prevented by a slight change in hormonal balance of the endometrium, hence providing successful contraception (Scommegna et al., 1970).

MATERIALS AND METHODS

Experimental Design and Animals

A completely randomized experiment involving 20 female beagles (Canis familiaris) of approximately the same size and weight were used to evaluate an intravaginal drug delivery device. Seventeen females with normal and known estrous cycles were obtained from the Gaines' Gerontology Beagle Colony at Iowa State University. An earlier experiment involving Control, High and Medium dietary protein levels utilized the same females that were used in this study. Three additional female beagles with normal estrous cycles were brought into this colony, 24 May 1978, from Ridglan Farms.[1]

Colony estrous records were evaluated and experimental subjects were selected on the basis of regular estrous cycles. These animals were then randomly assigned to treated (15 females) or control (5 females) groups. An additional 10 females within the colony were monitored for cycle regularity throughout the study and for accuracy of estrus prediction.

Test animals receiving delivery devices had the devices placed intravaginally approximately one month before the

[1]Ridglan Research Farms, Inc., 301 West Main Street, Mt. Horeb, Wisconsin.

expected estrus. Each delivery device remained intravaginally for a three month period and was then removed. Fifteen females were treated with mibolerone in the delivery device and 5 females were given "placebo" delivery devices.

Housing for the Beagles

All animals were housed in the facilities provided for the Gerontology Beagle Colony. Prior to 1 November 1978, they were kept in 5' by 10' wire enclosed pens within an unused dairy barn. Each pen contained a "dog house", which served as a sleeping and resting area for the animals. The floor of each "dog house" was triangular shaped (4' by 4' by 5.6') and had an 8" support leg at each corner. Sides of the "dog house" were 24" high, capped with a flat roof bearing the same dimensions as the floor. Food and water were available continuously in each pen. All female beagles were moved from the dairy barn facility on 1 November 1978 to a newly constructed building designed for the colony. Pens in the new building were 4' by 8' with food and water available continuously. Individual pens contained 3 or 4 beagles.

Drug Diffusion Rate of the Delivery Device

A number of delivery designs were discussed with Drs. Evans[1] and Hembrough[2] before the evolution of the slow-releasing drug-delivery device (Figure 1) used in this study. In tests by investigators at the Upjohn Company[3], silastic capsules had been used to deliver mibolerone subcutaneously. These capsules were found unsatisfactory in the delivery control of mibolerone. This author was unable to acquire information regarding mibolerone diffusion across the silastic membranes. As a result, accurate mibolerone diffusion rates through silastic membranes were unknown to the author at the beginning of this study. The theoretical estimate for the mibolerone diffusion rate was assumed to be equal to that reported for testosterone, 317 µg/24 hrs/100 mm^2/0.1 mm (Kincl et al., 1968).

The oral mibolerone dosage for the beagle is 30 µg/day to prevent estrus. It was calculated (based upon the above equation for testosterone) that a silastic membrane 1 mm thick with a 20 mm^2 surface area should deliver 6 µg of mibolerone/day. The spring pressure constructed into the

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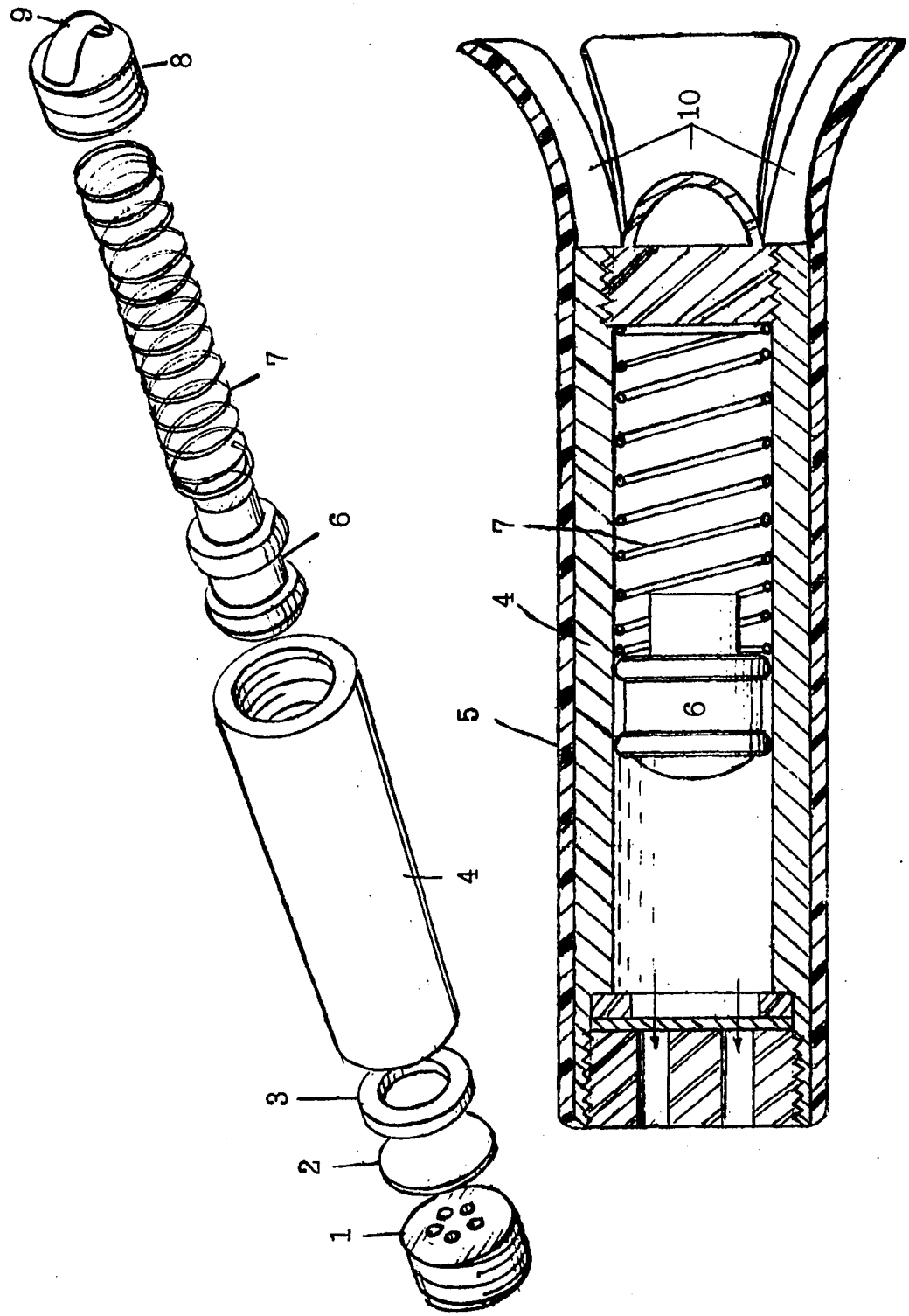
[1]Lawrence E. Evans, D.V.M., Ph.D., Department of Veterinary Clinical Science, Iowa State University, Ames, Iowa.

[2]Frederick Hembrough, D.V.M., Ph.D., Department of Veterinary Pharmacology and Physiology, Iowa State University Ames, Iowa.

[3]James Sokolowski, D.V.M., Reproduction Research, The Upjohn Company, Kalamazoo, Michigan.

Figure 1. The schematic design of the intravaginal slow-releasing drug-delivery device

1) Delivery end of the device
2) Dacron re-enforced silastic membrane
3) Retaining ring
4) Cylindrical housing
5) Medical grade silastic tubing covering the cylindrical housing
6) Rubber plunger
7) Steel coiled spring
8) Plug, sealing nondelivery end
9) Retrieval ring, when looped through the walls of housing, helps retain steel coiled spring
10) Silastic "fingers"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delivery device was assumed to have some effect on mibolerone diffusion. The pressure created in the delivery device was known to exceed 800 mm Hg (Table 1). As a result, the decision was made to consider the spring pressure effect on diffusion as a factor of 10, until proven otherwise. The 6 µg was then multiplied by 10 to take into consideration the spring pressure constructed into the device. So, in theory, the delivery device (U.S. Patent application No. 124, 542) for this study was designed to constantly deliver approximately 60 µg of mibolerone/day, double the normal effective oral dose.

Delivery Device Construction

The drug delivery device was hand constructed and conformed to the design shown in Figure 1. A 3 ml disposable, nontoxic, nonpyrogenic, polypropylene syringe[1] was cut down to 2.5 ml for the cylindrical housing. The delivery end was melted, flattened and cooled. Then 5, 1 mm diameter holes were drilled through its face. The inside face of the delivery end was then covered with an 8 mm diameter by 1 mm thick dacron re-enforced silastic sheeting[2] held in

[1] B-D Disposable 3 cc Syringe, Becton-Dickinson Division, Becton, Dickson and Company, Rutherford, New Jersey.

[2] Re-enforced Silastic Sheeting No. 501-7, Dow-Corning Chemical Company, Midland, Michigan.

place with a plastic retaining ring (I.D. 5 mm). Eight-tenths ml of 0.00876 grams of mibolerone crystals in 95% ethanol was then added to the chamber and secured in place by the original plunger, which was modified by shortening the plastic stem to fit into the new housing. A steel coil spring was placed behind the plunger and locked into the chamber by looping the retrieval ring through the housing. Scraps of polypropylene, cut earlier from the syringe, were melted and used to seal the end of the syringe containing the retrieval ring. After cleansing with 75% ethanol, the entire delivery device was inserted into an 8 cm piece of sterile silastic tubing[1]. Excess tubing was allowed to extend beyond the retrieval ring at the nondelivery end. The tubing beyond the housing was then cut into quarters (see design in Figure 1), creating 4 silastic "fingers" which served to retain the delivery device within the vagina. The tubing was attached to the housing at the base of the retrieval ring by silicone-rubber adhesive[2], which required 12 hours to cure. The device was cold sterilized with 75% ethanol as the final step in preparing the device for insertion into the vagina.

[1]Medical Grade Silastic Rubing (I.D. 3/8", O.D. 1/2"), Dow-Corning Chemical Company, Midland, Michigan.

[2]Medical Grade Silastic Adhesive No. 891, Dow-Corning Chemical Company, Midland, Michigan.

Delivery Device Internal Pressure

The slow-releasing drug-delivery device (Figure 1) included a compression spring which exerted pressure on the mibolerone-ethanol solution contained within the device. Pressure was measured in 4 of the delivery devices with a Statham transducer[1] and recorded on a Beckman polygraph[2]. In all 4 of the delivery devices, the initial pressure was measured with 0.8 ml of 95% ethanol in each of the devices. In 2 of these delivery devices, the pressure was measured with 0.2 ml of ethanol remaining and this was considered the end pressure.

Preparation of the Drug Solution

The drug solution was prepared by weighing (on an analytical balance[3]) 0.05475 grams of mibolerone crystals. The crystals were then dissolved in 5 ml of 95% ethanol. Each delivery device used in the mibolerone treated animals contained 0.8 ml of this solution or a similarly prepared solution.

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[1]Statham P23dB, Statham Instrument, Inc., Oxnard, Calif.

[2]Beckman R611 Dynograph, Beckman Instrument, Inc., Schiller Park, Illinois.

[3]Sartorius Balance Model No. 2400, Sartorius Balances Division, Brinkmann Instruments Inc., Westbury, New York.

Delivery Device Insertion and Removal

Insertion of the drug delivery device was a very simple process. The conscious animal was maintained in a standing position by one person placing one hand under the abdomen and the other hand holding the tail erect. A second person clamped the retrieval ring on the delivery device with a Bozeman uterine dressing forcep[1] (Figure 2) and dipped the delivery end into a jar of nitrofurazone dressing. The nitrofurazone was allowed to lightly cover one inch of the delivery end. The vulva of the beagle was then cleansed with a solution of 75% ethanol and 25% chlorhexidine hydrochloride. Next, with the fingers of one hand the labia were separated and the delivery device inserted, delivery end first (Figure 3). Gentle dorsocephalad pressure moved the device to a position dorsal to the pubic bone. The directional pressure was then changed to cephalad, moving the device to a position cranial to the pubic bone. The forcep was then released and removed. The same insertion procedure was accomplished by one person with five of the animals, without restraint.

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[1]Bozeman Uterine Dressing Forcep, Arista Surgical Supply Company, Inc., New York, New York.

Figure 2. Uterine dressing forcep clamped to the retrieval ring of the intravaginal drug delivery device

Figure 3. The drug delivery device was easily and gently inserted into the vaginal canal

The procedure for removing the device underwent several changes during the experimental period. In the first two animals, the delivery devices each had a 3" piece of 2-0 suture material attached to the retrieval ring (Figure 4). One female was able to remove the device herself, so attachment of suture material was discontinued. As an alternative procedure, the uterine dressing forcep was used unsuccessfully in several attempts to clasp the delivery device and pull it out. This method presented the danger of clamping the vaginal mucosal lining along with the delivery device hence tearing or crushing the tissue. A third removal technique involved hooking the delivery device retrieval ring with a modified Covault spay hook[1]. This method was met with considerable resistance from the animal, caused some vaginal bleeding and took approximately 10 minutes under the best of conditions. An improved removal procedure (Figures 6-11), used on 3 animals, has been most effective and practical. An anoscope[2] (Figure 5), with a 127 mm speculum length and a 14 mm aperture was inserted into the vagina to allow easy viewing of the retrieval ring (Figure 9). The spay hook was inserted through the anoscope and the retrieval

[1]Covault Spay Hook, Arista Surgical Supply Company, Inc., New York, New York.

[2]Long Speculum Anoscope No. 39514, Welch Allyn, Inc., Skaneateles Falls, New York.

Figure 4. The initial drug delivery device, used in this study, incorporated a piece of suture material to assist removal

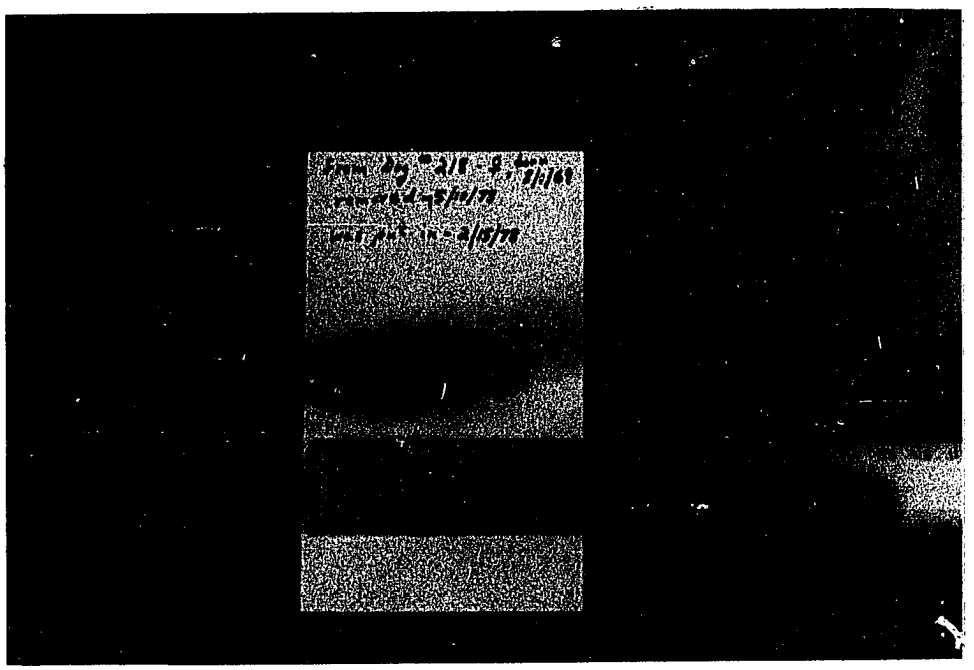


Figure 5. The long speculum anoscope and spay hook were valuable tools utilized in the removal of the delivery device

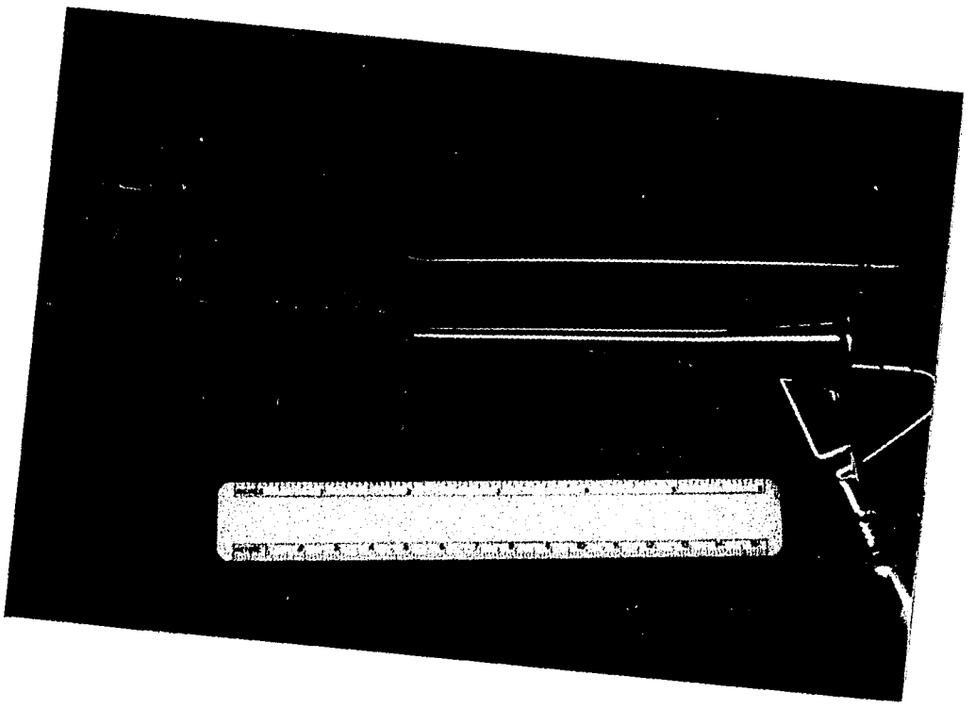


Figure 6. Beagle X03 had received a drug delivery device 4 weeks earlier (note - no vaginal discharge)

Figure 7. Delivery device removal from Beagle X03. Insertion of the anoscope into the vaginal canal

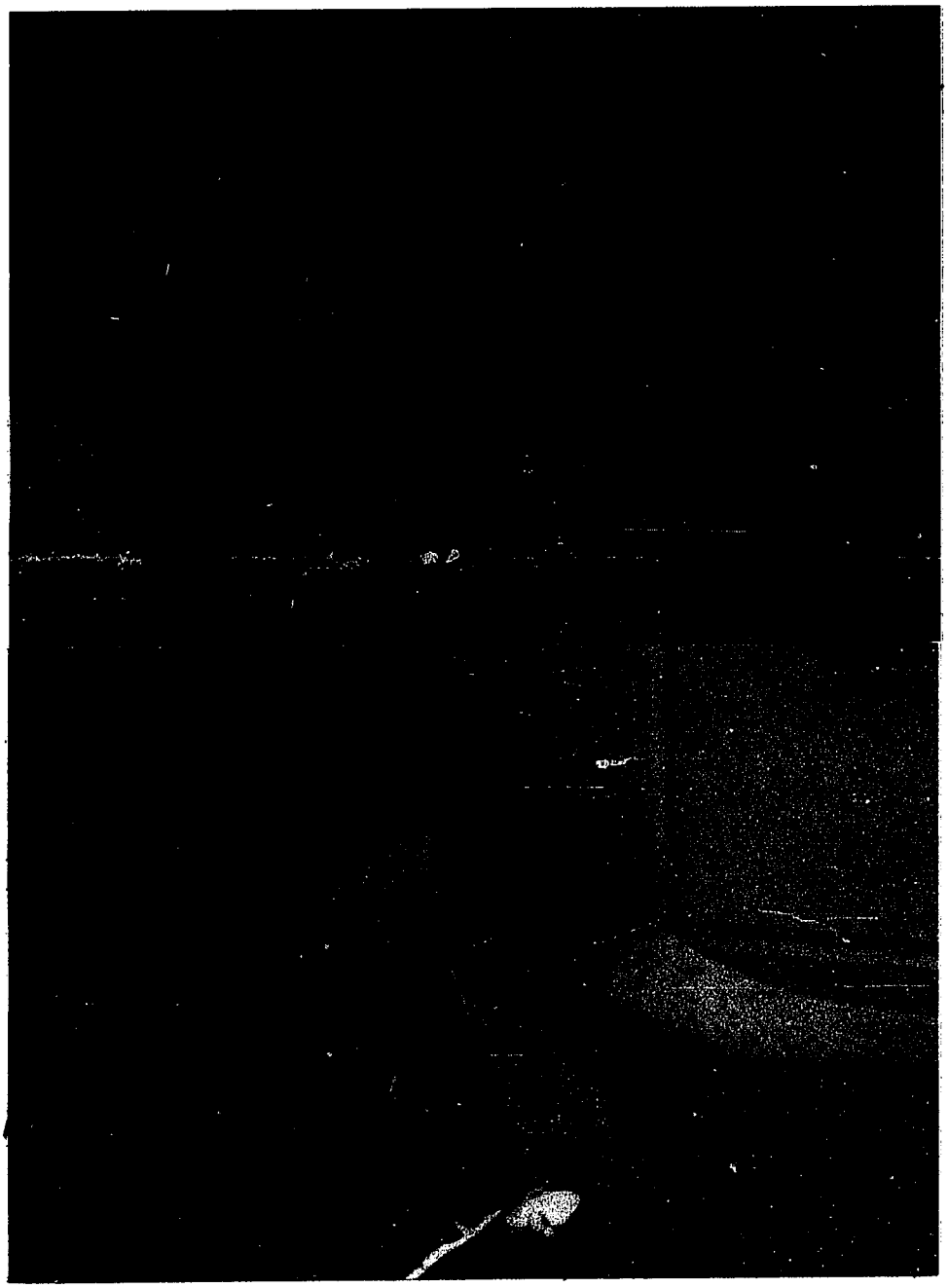


Figure 8. Delivery device removal from Beagle X03. The spay hook was inserted through the anoscope and attached to the retrieval ring of the delivery device

Figure 9. Delivery device removal from Beagle X03. The retrieval ring on the delivery device was easily seen through the anoscope, permitting quick attachment of the spay hook

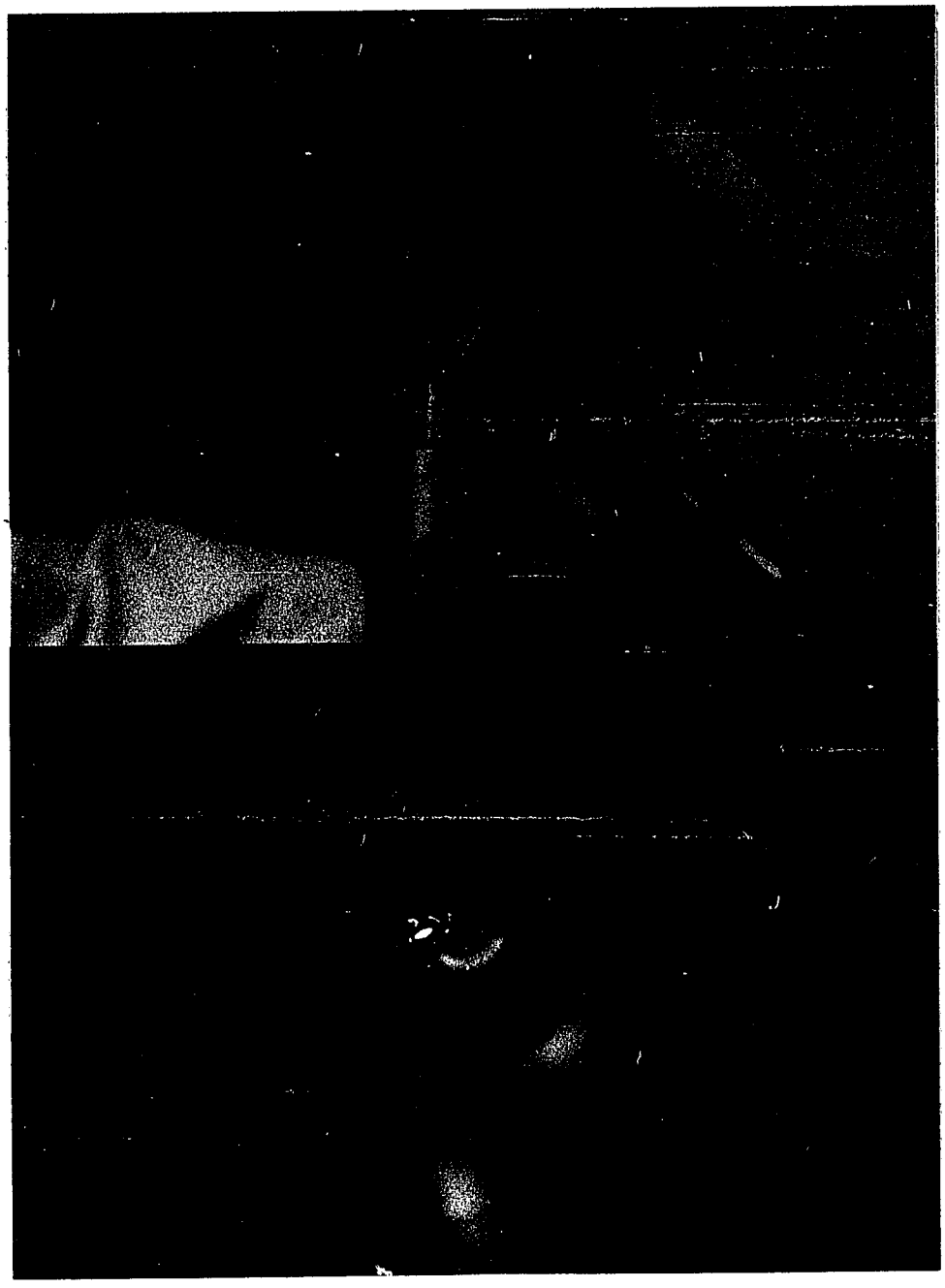
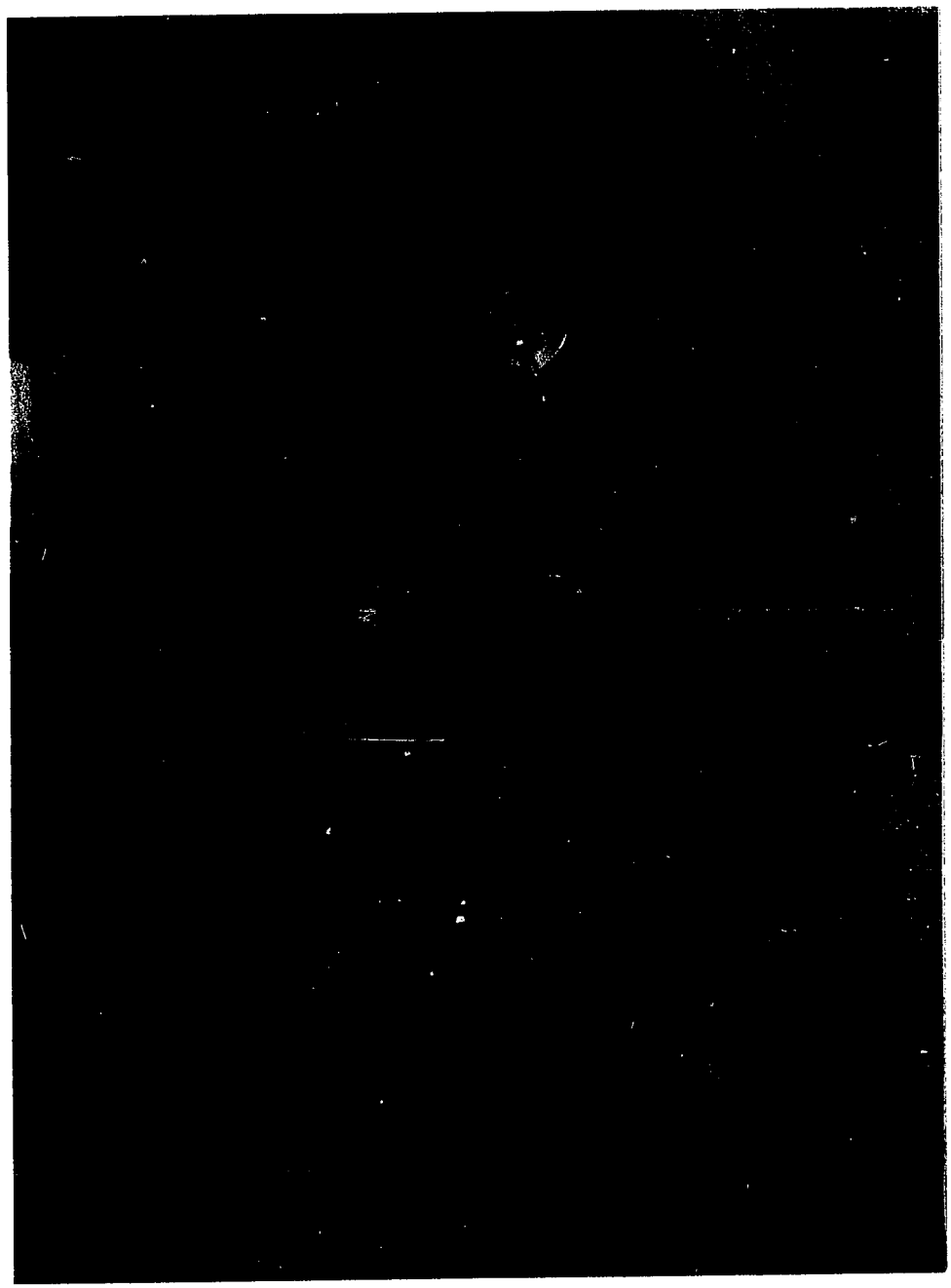


Figure 10. Delivery device removal from Beagle X03. The delivery device was held firmly against the end of the anoscope as removal began

Figure 11. Delivery device removal from Beagle X03. The delivery device was removed while held firmly against the end of the anoscope



ring hooked. The delivery device was held firmly against the end of the anoscope as both were removed from the vagina. This procedure was accomplished by one individual on a fully conscious animal in less than one minute.

Plasma Collection for the Analysis of Mibolerone and/or Progesterone Levels

Blood samples (approximately 8 ml/sample) were taken once every two weeks during the test period. Blood was collected from the right or left cephalic vein into heparinized vacutainers[1] (10 ml containers) via a 20 gauge bleeding needle. The blood was then centrifuged[2] at 2100 rpm for 15 minutes and the plasma was pipetted, transferred to 3.5 ml plastic vials, and frozen until radioimmunoassayed for mibolerone or progesterone.

Mibolerone Assay

Frozen plasma samples were sent to the Upjohn Company[3] for analysis of mibolerone levels. The assay used a double antibody radioimmunoassay technique as described by Krzeminski et al. (1978). The sensitivity of the assay

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[1]B-D Heparinized Vacutainer, 10 ml volume, Becton-Dickinson Division, Becton, Dickinson and Company, Rutherford, New Jersey.

[2]Model CL, IEC Clinical Centrifuge, Damon/IEC Division, Needham Heights, Massachusetts.

[3]Jenaay M. Brown (D.V.M), Reproduction Research, The Upjohn Company, Kalamazoo, Michigan.

was 1.8 parts per billion.

Progesterone Assay

The radioimmunoassay procedure for progesterone was initiated by pipetting 100 µl of thawed plasma sample and 400 µl of Tris buffer into a test tube. To this tube, 4.5 ml of petroleum ether was added. The test tube was then tightly capped and shaken for 15 minutes. Following the period of shaking, the test tube was placed in a dry ice-methanol bath until the water layer in the tube froze. The petroleum ether phase was decanted into another test tube which then was placed in a 40°C water bath. The petroleum ether evaporated and the test tube was removed from the bath. Next, 100 µl of Tris buffer was added to the test tube and vortexed vigorously. The progesterone extraction from the plasma was completed at this point. The assay continued with 0.4 ml of the antibody and 0.1 ml of the tracer (^{3}H-progesterone) simultaneously added to the test tube and gently vortexed. The test tube was covered with paraffin and incubated overnight in a "cold room".

The final stages of the assay were completed by adding 0.2 ml of charcoal-dextran with vortexing of the test tube. Fifteen minutes later the test tube was centrifuged at 3000 rpm for 10 minutes. Next, a 0.5 ml sample of the assay

supernatant along with 4.5 ml of a scintillation cocktail was pipetted into a scintillation vial. Radioactivity for the sample was monitored on the tritium channel of a liquid scintillation counter and recorded. The resulting sample counts were compared with progesterone standards that were assayed simultaneously with this group of samples.

The complete procedure for progesterone analysis is included in Appendix B.

Vaginal Smears to Determine the Phase of the Estrous Cycle

Smears of cells from the vaginal mucosa were taken weekly from each animal throughout the test period and for 4 weeks beyond the end of the test period. First, the vulva of the female was cleansed with a 75% ethanol and 25% chlorhexidine hydrochloride solution. The labia then were separated with one hand and a sterile cotton swab was introduced into the dorsal aspect of the labial commissure with the other hand. The swab was gently inserted (approximately 10 cm) through the vaginal vestibule, past the pelvic brim and along the craniodorsal aspect of the vagina. The swab was rotated to free and collect cells from the mucosal lining and then was gently withdrawn. After withdrawal, the swab was rolled onto a precleaned microscope slide. The vaginal

cells then were stained with Wright's stain in an Ames Hema-Tek slide stainer[1] and cell types were evaluated microscopically.

Daily Physical Examination

To assist in the evaluation for estrus during the test period, daily physical examinations for vaginal discharge and labial swelling were performed. Behavior also was observed during this daily examination. Digital stimulation was applied to the dorsal aspect of the lumbar area to see if a breeding stance would be taken. Observations were also made to determine whether the treated female was "teasing" other females in the pen and whether the other females were trying to mount the treated female. Animals were checked daily for ocular discharge that is known to occur with excessive doses of mibolerone.

Estrus Resumption after Treatment

Each treated female was checked for estrus 3 times weekly, following treatment termination. To confirm suspected estrus, vaginal smears and/or testing of plasma progesterone levels were evaluated.

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[1]Ames Hema-Tek Slide Stainer, Miles Laboratory, Inc., Elkhart, Indiana.

Vaginal Examination with a Cystoscope

A cystoscope[1] was used to examine the vaginal tract of three females, containing a delivery device, to check for the presence of any excessive exudate, inflammation and/or hyperemia. Two beagles were anesthetized with thiamylal sodium (6 mg/lb) and their vaginal tracts were visually inspected with the use of the cystoscope. The delivery device was removed using an anoscope (Figures 6-11) and the vagina re-inspected using the cystoscope. Photographs were taken with a 35 mm Pentax camera[2] attached to the cystoscope. A third beagle was examined using the cystoscope and the anoscope, while fully conscious throughout the examination. Photographs were taken with a 35 mm Olympus camera[3] while viewing through the anoscope.

Body Weight Data Collection

Androgens are known to be anabolic (tissue building), so body weights were obtained weekly for each animal in order to evaluate whether or not this was a problem in mibolerone

[1]Model FCB 1000 Fiber Optic, American Cystoscope Makers Inc., Pelham Manor, New York.

[2]Model ES Pentax Camera, Honeywell, Littleton, Colorado.

[3]Model OM-1 Olympus Camera with 50 mm lens, Olympus Optical Co., Ltd., Tokyo, Japan.

treatment. Weights were recorded beginning one month before and continued for one month beyond the test period. Weight was recorded to the nearest half pound.

Statistics

A standard t-test was computed for progesterone levels to determine if there was a difference between treated and nontreated animals.

An analysis of variance was computed for body weight gain and loss data. The analysis was undertaken to provide answers for the following questions:

1) Was there a difference in weight gain or loss between mibolerone treated animals versus nontreated animals?

2) Was there a difference in weight gain or loss between mibolerone treated versus nontreated females on different levels of the protein diet?

3) Did age have an effect on weight gain or loss in treated versus nontreated animals?

RESULTS

Delivery Device Internal Pressure

The internal pressure recorded from within four delivery devices ranged from 800 mm Hg to 870 mm Hg. Two of the devices had both initial and end delivery pressures recorded, the other two had just the initial delivery pressure recorded.

Table 1. Delivery device internal pressure (in mm Hg)

Delivery device	Initial pressure	End pressure
1	800	800
2	870	870
3	800	-
4	840	-

Body Weight

Age of the beagles, dietary protein, treatment, initial body weight and body weight at the end of six weeks are summarized in Table 2. The data in Table 2 were used for the analysis of variance to evaluate treatment effects on body weight. A six-week time period was chosen because the delivery devices were known to contain a portion of the

Table 2. The data for treatment effect on body weight

Beagle	Age (years)	Dietary protein level	Treatment	Initial weight (lbs)	Six week weight (lbs)
218	9	control	mibolerone	25.0	22.0
427	10	control	mibolerone	21.5	21.0
S62	5	medium	mibolerone	25.5	24.5
YQI1	7	control	mibolerone	29.5	27.0
PQY2	6	control	mibolerone	31.0	30.0
VDU3	5	control	mibolerone	24.0	25.0
867	7	control	mibolerone	30.5	30.0
S57	5	high	mibolerone	26.0	27.0
PO11	7	control	mibolerone	20.0	20.0
S54	5	high	mibolerone	24.0	24.5
U17	4	high	mibolerone	26.0	26.5
S42	5	medium	mibolerone	21.0	21.0
U06	5	medium	mibolerone	32.0	31.0
U09	4	medium	mibolerone	28.0	27.0
X02	3	high	mibolerone	36.0	38.0
X03	3	high	mibolerone	25.0	25.0
S31	5	medium	control	18.0	18.0
S07	5	medium	control	19.0	18.0
P014	7	control	control	30.5	30.0
144	6	control	control	26.0	30.0
262	14	control	control	22.0	24.0
P023	7	control	control	30.5	34.0
P018	7	control	control	19.0	17.0
P019	7	control	control	17.0	15.0
S61	5	medium	control	27.0	27.0
S13	5	medium	control	18.5	18.0
U03	5	medium	control	19.0	17.0
X04	3	medium	control	23.0	24.0
150	6	control	control	21.0	22.0
155	6	control	control	26.0	26.0
S23	5	high	control	23.0	21.0
U10	4	high	control	27.0	28.0
S34	5	high	control	20.0	20.5
P036	6	high	control	26.0	25.0
P035	6	high	control	20.0	19.0

mibolerone and ethanol solution at that time. The intial weight of each beagle and the weight at the end of the six-week treatment period are also reported in Table 2. Overall, the mean body weight change, after six weeks of treatment, was -0.34 pounds for mibolerone treated beagles and +0.05 pounds for the control beagles.

The initial analysis of variance evaluated differences in body weight changes, after six weeks, between the mibolerone treated and control treated females. The following results were obtained:

Source	Degrees of freedom	Sum of squares	Mean square	F-value
Treatment	1	1.36	1.36	0.57
Error	33	79.31	2.40	
TOTAL	34	80.67		

These results indicate there was no significant ($P<0.05$) difference in body weight gain or loss between mibolerone treated and control treated female beagles.

Analysis of variance comparing the effect of the treatment, dietary protein level and the treatment and dietary protein level interaction provided the following results:

Source	Degrees of freedom	Sum of squares	Mean square	F-value
Model	5	17.70	3.54	1.63
Error	29	62.97	2.17	
TOTAL	34	80.67		
Treatment	1	1.36	1.36	0.63
Diet	2	2.69	1.34	0.62
Treatment-Diet Interaction	2	13.65	6.82	3.14

The dietary protein level and/or the treatment-diet interaction did not significantly ($P<0.05$) effect body weight.

Treatment and age and a treatment-age interaction were analyzed with an analysis of variance, and the following results were obtained:

Source	Degrees of freedom	Sum of squares	Mean square	F-value
Model	12	26.46	2.20	0.89
Error	22	54.21	2.46	
TOTAL	34	80.67		
Treatment	1	1.36	1.36	0.55
Age	7	20.26	2.89	1.17
Treatment-age Interaction	4	4.83	1.21	0.49

The results indicate that treatment and age or a treatment-age interaction had no significant ($P<0.05$) effect on body weight.

Cystoscopic Examination of the Vagina

A gross vaginal examination, with a cystoscope, was performed on three female beagles four weeks after the delivery device had been inserted. The vaginal canal appeared grossly normal in each beagle and it was noted that there was no excessive exudate, inflammation or hyperemia present (Figure 12). Following the delivery device removal, there was a small amount of bleeding into the vaginal lumen of two females. This bleeding was thought to be caused by the examination and delivery device removal procedures.

Mibolerone Radioimmunoassay

Mibolerone was detected in none of the 98 plasma samples which were assayed. The radioimmunoassay used in this study had a lower range of sensitivity of 1.8 parts per billion.

Figure 12. A view within the vaginal canal of Beagle X03, 4 weeks after delivery device insertion (note - no excessive exudate, inflammation or hyperemia)

Monitoring During Treatment to Determine the Phase of the Estrous Cycle

Progesterone levels, vaginal cell smears and discharge and vulvar swelling were monitored during the treatment period to ascertain estrous phase for each animal during the treatment period.

Radioimmunoassay for progesterone was completed on 204 plasma samples (Table 3). The majority of these samples were from females that were treated with mibolerone released by the delivery device. The original intention was to assay the plasma samples of the five control treated beagles during diestrus; however, one sample was mistakenly taken when the animal was in late estrus. The beagle (P036) in late estrus had 9.18 ng progesterone/ml of plasma. The 4 diestrual beagles (P023, S61, S42 and S43) had a mean peak of 24.7 ng progesterone/ml of plasma. The progesterone levels in most of the females treated with the mibolerone remained at levels expected during anestrus (0.0 ng to 1.0 ng/ml of plasma). However, 3 of the beagles (P011, U06 and X02) treated with mibolerone had an unexpected rise in progesterone level during the treatment period (Table 3 and Figure 13). The rise in progesterone level in these 3 animals did not attain the levels normally seen during diestrus but averaged 7.4 ng progesterone/ml of plasma at their peak. The vaginal cell smears for these 3 animals

Figure 13. Plasma progesterone levels during treatment

(Control - illustrates the normal expected plasma progesterone level increases during estrus and diestrus,
Group A - were drug treated animals (n=12) which demonstrated no increases in progesterone levels during the treatment period,
Group B - were drug treated animals (P011, U06 and X02) which demonstrated some increase in progesterone levels during the treatment period.

Note - Groups A and B demonstrated no vaginal discharges and the vaginal smears indicated anestrus during the treatment period)

Plasma progesterone levels during treatment

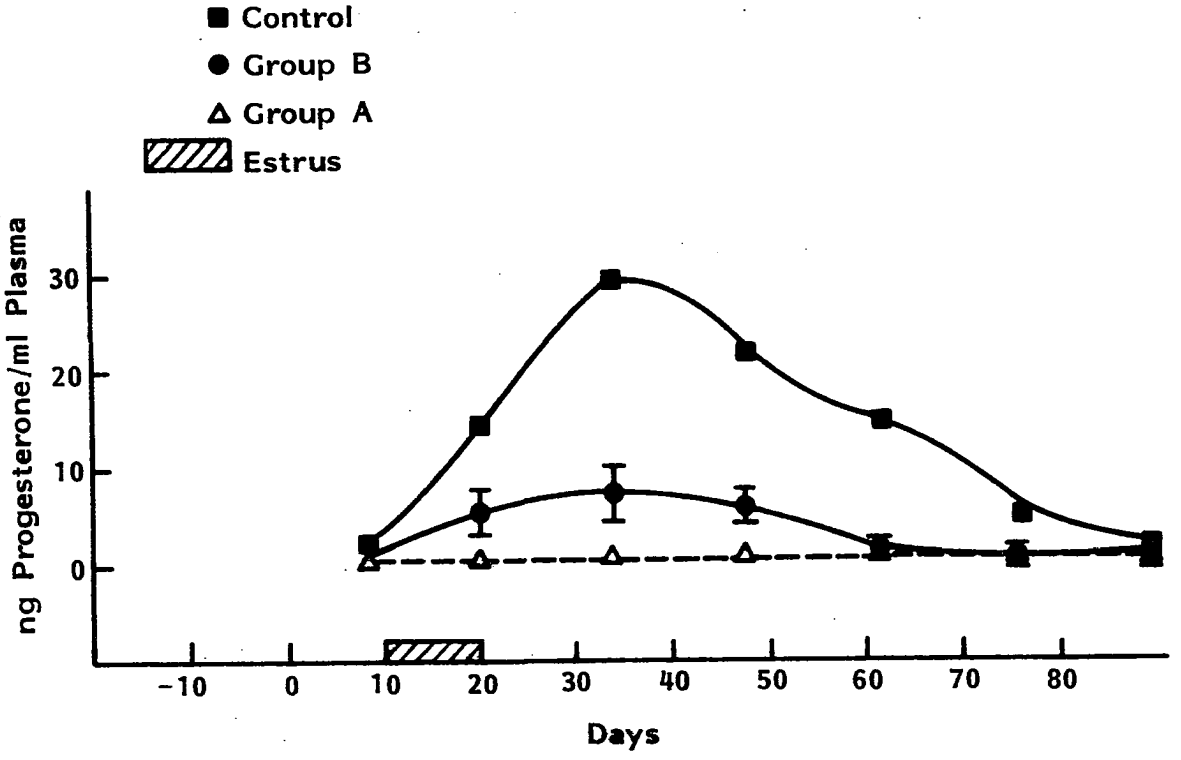


Table 3. Progesterone levels and phase of the estrous cycle

Beagle	Date	ng progesterone/ ml of plasma	Estrous cycle stage as indicated by vaginal cell smear
218	02-15-78	0.37 Treatment begun	Anestrus
	03-01-78	TLH[a]	Anestrus
	03-15-78	TLH	Anestrus
	03-16-78	Expected estrus	
	03-29-78	TLH	Anestrus
	04-12-78	TLH	Anestrus
	04-26-78	TLH	Anestrus
	05-10-78	TLH Treatment stop	Anestrus
	01-03-79	16.50	Late estrus
427	02-15-78	TLH Treatment begun	Anestrus
	03-01-78	TLH	Anestrus
	03-15-78	TLH	Anestrus
	03-20-78	Expected estrus	
	03-29-78	TLH	Anestrus
	04-12-78	TLH	Anestrus
	04-26-78	TLH	Anestrus
	05-10-78	TLH Treatment stop	Anestrus
	05-24-78	TLH	Anestrus
	06-07-78	TLH	Proestrus
	06-21-78	19.55	Diestrus
	07-05-78	26.03	Diestrus
	07-19-78	12.31	Diestrus
	01-03-79	29.83	Diestrus
867	02-15-78	0.28 Treatment begun	Anestrus
	03-01-78	TLH	Anestrus
	03-15-78	TLH	Anestrus
	03-17-78	Expected estrus	
	03-29-78	TLH	Anestrus
	04-12-78	TLH	Anestrus
	04-26-78	TLH	Anestrus
	05-10-78	TLH Treatment stop	Anestrus
	05-24-78	TLH	Anestrus
	06-07-78	TLH	Anestrus
	06-21-78	TLH	Anestrus
	07-05-78	1.92	Estrus
	12-20-78	35.36	Diestrus

[a]TLH = too little hormone, meaning that less than 0.25 ng/ml was present.

Table 3 (Continued)

Beagle	Date	ng progesterone/ ml of plasma	Estrous cycle stage as indicated by vaginal cell smear
S62	05-10-78	TLH Treatment begun	Anestrus
	05-24-78	TLH	Anestrus
	06-07-78	TLH	Anestrus
	06-20-78	Expected estrus	
	06-21-78	TLH	Anestrus
	07-05-78	TLH	Anestrus
	07-19-78	TLH	Anestrus
	08-02-78	TLH Treatment stop	Anestrus
	08-15-78	TLH	Anestrus
	08-30-78	0.88	Anestrus
	09-13-78	11.24	Estrus
	09-27-78	19.14	Diestrus
	03-08-79	22.92	Diestrus
YQ11	05-24-78	TLH Treatment begun	Anestrus
	06-07-78	TLH	Anestrus
	06-21-78	TLH	Anestrus
	06-30-78	Expected estrus	
	07-05-78	0.30	Anestrus
	07-19-78	TLH	Anestrus
	08-02-78	TLH	Anestrus
	08-15-78	TLH Treatment stop	Anestrus
	08-30-78	0.34	Anestrus
	09-13-78	18.82	Metestrus - Late estrus
	09-27-78	16.43	Diestrus
	10-11-78	20.53	Diestrus
	03-08-79	2.38	Early estrus
	03-22-79	23.18	Diestrus
PQY2	04-24-78	TLH Treatment begun	Anestrus
	06-07-78	TLH	Anestrus
	06-21-78	TLH	Anestrus
	06-28-78	TLH	Anestrus
	06-28-78	Expected estrus	
	07-05-78	TLH	Anestrus
	07-19-78	TLH	Anestrus
	08-02-78	TLH	Anestrus
	08-15-78	TLH Treatment stop	Anestrus
	08-30-78	0.33	Anestrus
	09-13-78	15.29	Estrus
	09-27-78	18.84	Diestrus
	10-11-78	7.87	Diestrus
	10-25-78	TLH	Anestrus
	12-06-78	27.28	Diestrus

Table 3 (Continued)

Beagle	Date	ng progesterone/ ml of plasma	Estrous cycle stage as indicated by vaginal cell smear
VDU3	05-24-78	TLH Treatment begun	Anestrus
	06-07-78	TLH	Anestrus
	06-21-78	TLH	Anestrus
	07-05-78	TLH	Anestrus
	07-07-78	Expected estrus	
	07-19-78	TLH	Anestrus
	08-02-78	TLH	Anestrus
	08-15-78	TLH Treatment stop	Anestrus
	08-30-78	TLH	Anestrus
	09-13-78	TLH	Anestrus
	09-27-78	TLH	Anestrus
	11-18-78		Estrus
	12-06-78	22.32	Diestrus
S57	09-13-78	TLH Treatment begun	Anestrus
	09-27-78	1.43	Anestrus
	09-30-78	Expected estrus	
	10-11-78	TLH	Anestrus
	10-25-78	TLH	Anestrus
	11-07-78	TLH	Anestrus
	11-22-78	TLH	Anestrus
	12-06-78	TLH Treatment stop	Anestrus
	12-20-78	TLH	Anestrus
	01-03-79	TLH	Anestrus
01-19-79		3.92	Late proestrus
P011	09-27-78	TLH Treatment begun	Anestrus
	10-11-78	TLH	Anestrus
	10-15-78	Expected estrus	
	10-25-78	TLH	Anestrus
	11-07-78	TLH	Anestrus
	11-22-78	5.53	Anestrus
	12-06-78	6.74	Anestrus
	12-20-78	6.63 Treatment stop	Anestrus
	01-03-79	2.38	Anestrus
	01-19-79		Anestrus
	01-31-79	1.74	Anestrus
	10-20-79		Estrus

Table 3 (Continued)

Beagle	Date	ng progesterone/ ml of plasma	Estrous cycle stage as indicated by vaginal cell smear
S54	09-27-78	0.39 Treatment begun	Anestrus
	10-11-78	TLH	Anestrus
	10-25-78	TLH	Anestrus
	11-06-78	Expected estrus	
	11-07-78	TLH	Anestrus
	11-22-78	TLH	Anestrus
	12-06-78	TLH	Anestrus
	12-20-78	TLH Treatment stop	Anestrus
	01-03-79	TLH	Anestrus
	01-19-79	0.35	Early proestrus
	01-31-79	26.42	Diestrus
U17	09-27-78	TLH Treatment begun	Anestrus
	10-11-78	TLH	Anestrus
	10-20-78	Expected estrus	
	10-25-78	TLH	Anestrus
	11-07-78	0.29	Anestrus
	11-22-78	0.25	Anestrus
	12-06-78	TLH	Anestrus
	12-20-78	TLH Treatment stop	Anestrus
	01-03-79	TLH	Anestrus
	01-19-79	1.40	Proestrus
	01-31-79	24.47	Diestrus
	02-15-79	19.17	Diestrus
U06	02-15-79	TLH Treatment begun	Anestrus
	03-08-79	TLH	Anestrus
	03-20-79	Expected estrus	
	03-22-79	8.58	Anestrus
	04-05-79	11.19	Anestrus
	04-17-79	6.86	Anestrus
	05-02-79	2.22	Anestrus
	05-16-79	0.90 Treatment stop	Anestrus
	06-04-79	TLH	Anestrus
	06-13-79	TLH	Anestrus
	06-27-79	TLH	Anestrus
U09	03-08-79	0.56 Treatment begun	Anestrus
	03-22-79	TLH	Anestrus
	04-05-79	TLH	Anestrus
	04-10-79	Expected estrus	
	04-17-79	TLH	Anestrus
	05-02-79	TLH	Anestrus
	05-16-79	TLH	Anestrus
	06-04-79	TLH Treatment stop	Anestrus
	06-13-79	TLH	Anestrus
	06-27-79	TLH	Anestrus
	07-25-79		Estrus

Table 3 (Continued)

Beagle	Date	ng progesterone/ ml of plasma	Estrous cycle stage as indicated by vaginal cell smear
X02	03-08-79	TLH Treatment begun	Anestrus
	03-22-79	2.33	Anestrus
	04-01-79	Expected estrus	
	04-05-79	4.40	Anestrus
	04-17-79	3.71	Anestrus
	05-02-79	2.62	Anestrus
	05-16-79	1.71	Anestrus
	06-04-79	TLH Treatment stop	Anestrus
	06-13-79	TLH	Anestrus
	06-27-79	TLH	Anestrus
X03	03-08-79	TLH Treatment begun	Anestrus
	03-22-79	TLH	Anestrus
	04-01-79	Expected estrus	
	04-05-79	TLH	Anestrus
	04-17-79	TLH	Anestrus
	05-02-79	TLH	Anestrus
	05-16-79	TLH	Anestrus
	06-04-79	TLH Treatment stop	Anestrus
	06-13-79	TLH	Anestrus
	06-27-79	TLH	Anestrus
	09-16-79		Estrus
P023	05-20-79	Expected estrus	
	06-04-79	25.34	Diestrus
	07-19-79	16.46	Diestrus
S61	07-29-78	Expected estrus	
	08-15-78	31.43	Diestrus
S42	04-12-78	TLH	Anestrus
	05-02-78	Expected estrus	
	05-10-78	20.14	Diestrus
	05-24-78	23.63	Diestrus
	12-06-78	TLH	Anestrus
	12-17-78	Expected estrus	
	12-20-78	11.20	Estrus
S43	12-06-78	18.33	Metestrus - Early Diestrus
	12-12-78	Expected estrus	
P036	09-27-78	TLH	Anestrus
	10-05-78	Expected estrus	
	10-11-78	9.18	Estrus

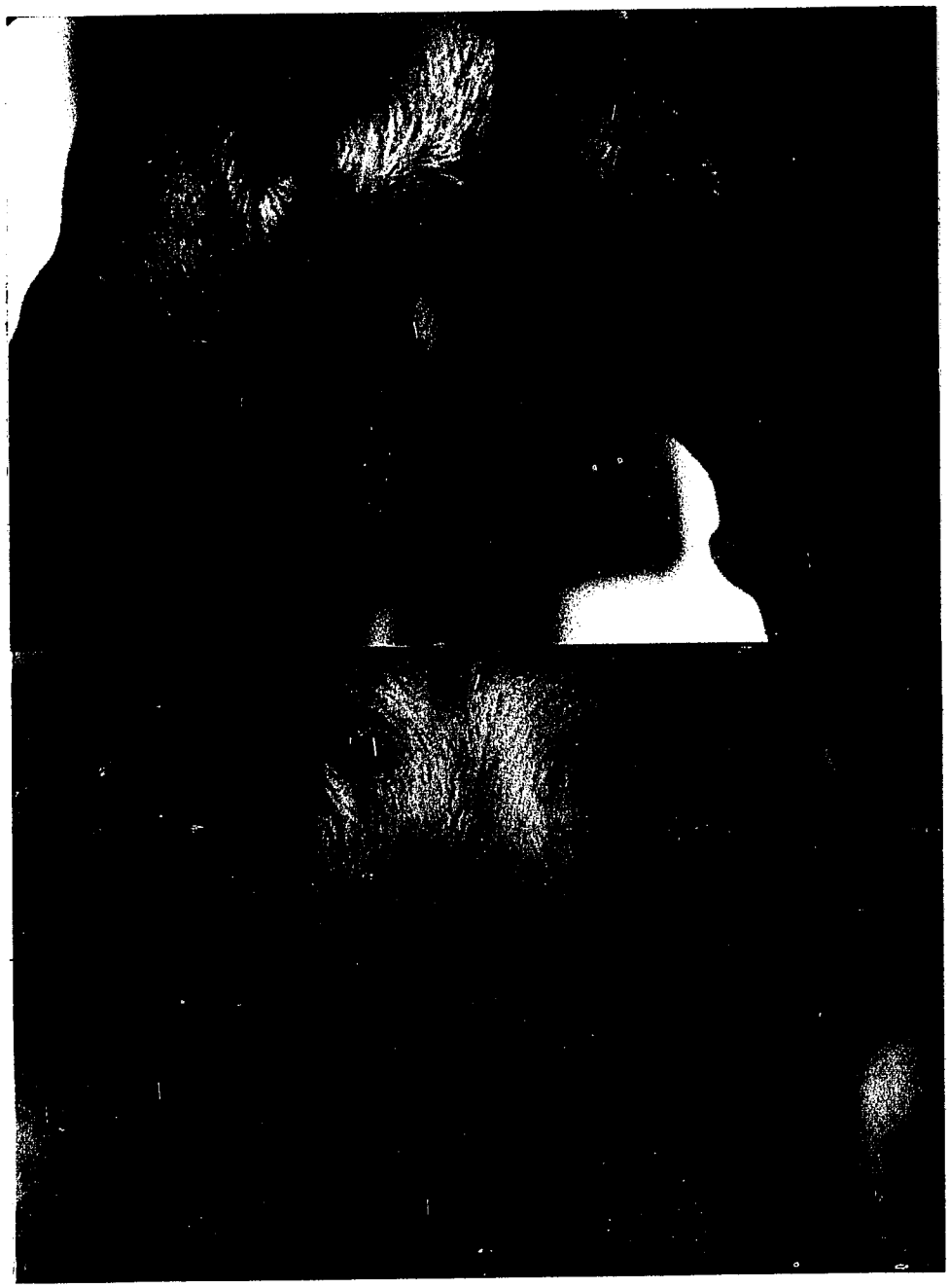
indicated an anestrus condition during this same period of progesterone elevation (Table 3). Also, gross examination during this same period revealed no vulvar swelling and no vaginal discharge. The vaginal smears, vulvar swellings and discharges for the control animals were in agreement with progesterone levels.

Gross Examination for Mibolerone Overdose

The mibolerone treated animals were examined daily for the presence of ocular and vaginal discharges and clitoral enlargement, phenomena which are known to be associated with a mibolerone overdose. Ocular discharge was occasionally seen in many of the beagles, treated and nontreated, which made it impossible to determine if the discharge was mibolerone related. Vaginal discharge and clitoral enlargement were not seen throughout the treatment period in animals treated with mibolerone. The general appearance of the vulva of two beagles, after six weeks of mibolerone treatment, can be seen in Figures 14 and 15.

Figure 14. Beagle VDU3 illustrates the appearance of the vulva 6 weeks after the delivery device was inserted (note - no vaginal discharge)

Figure 15. Beagle P011 illustrates the appearance of the vulva 6 weeks after the delivery device was inserted (note - no vaginal discharge)



Statistical Analysis of Progesterone Levels

A statistical analysis of progesterone levels did indicate a significant ($P<0.001$) difference in progesterone level means between the mibolerone treated and control beagles. (The calculations are reported in Table 4). The mean progesterone level for mibolerone treated females (during the

Table 4. Calculations for the statistical analysis of progesterone levels

	Mibolerone treated animals (m)	Control treated animals (c)
n =	15	4
Totals ΣX =	27.20	98.73
Means ($\bar{X}$) =	1.81	24.68
ΣX^2 =	193.20	2,524.33
$(\Sigma X)^2/n$ =	49.32	2,436.90
Sum of Squares $\Sigma x^2 = \Sigma X^2 - (\Sigma X)^2/n$ =	143.88	87.43
Degrees of freedom $n-1$ =	14	3
Pooled sample variance = $s^2 = \frac{x_m^2 + x_c^2}{n_m+n_c-2}$ =		13.61
Standard deviation between means = $s_{\bar{X}_m-\bar{X}_c} = \sqrt{s^2(\frac{n_m+n_c}{n_m n_c})}$ =		2.08
Calculated 't' = $\frac{n_m+n_c}{s_{\bar{X}_m-\bar{X}_c}}$ =		9.13
Table 't' value ($P<0.001$) =		3.96

treatment period) was 1.81 ng progesterone/ml of plasma and for control beagles was 24.68 ng progesterone/ml of plasma. The calculated t-value was 9.13 with 17 degrees of freedom and the table t-value was 3.96 (at $P<0.001$).

DISCUSSION

Intravaginal Drug Delivery for Estrus Control

The intravaginal route for delivering estrus controlling drugs has not been previously utilized in the canine. One reason this route may have been avoided by other investigators is that they may have had problems retaining an intravaginal device. In this study there was 100% retention of all delivery devices tested. The silastic "fingers" of the delivery device provided a very simple and satisfactory means of retaining the device within the vaginal canal of the beagle. Unfortunately, this same retaining design may not be applicable to animals other than the canine.

Unlike the ABC device (Hauge, 1974), the delivery device for the present study was neither designed nor intended to prevent penile insertion. Possibly, the intravaginal delivery device may hinder penile insertion, but it probably will not prevent it.

Cystoscopic Examination of the Vagina

Inflammation and infection are risks whenever a foreign body is placed in the vagina. For the present study, a medical grade silastic tubing was selected specifically to cover the delivery device in order to minimize the possibility

of intravaginal inflammation or infection.

Six of the beagles (427, 218, 867, PQY2, YQI1 and U06) receiving delivery devices had an occasional slight vaginal discharge during the first 2 weeks after device insertion. The vaginal discharges were serosanguineous, all of which cleared by the third week after delivery device insertion.

Three females had delivery devices re-inserted into the vaginal canal in order to gain an insight into the gross effect of the delivery device upon the vaginal mucosa. Delivery devices were re-inserted into the vagina of females U09 and X03, 6 months after the original treatment study. The devices remained intravaginally 4 weeks before the vaginal examination with the cystoscope. Another beagle (X02) had the delivery device re-inserted intravaginally 7 months after the mibolerone treatment and was examined with the cystoscope 3 weeks later. Beagle X03 did have a slight serosanguineous vaginal discharge for the first few days after the delivery device was re-inserted. Upon examination, no females were found to have excessive exudate, inflammation or vaginal bleeding. A view of the vaginal mucosa of beagle X02 can be seen in Figure 12.

The conclusion is that the delivery device was compatible with the vaginal mucosa, after a 2 to 3 week adjustment period, for the duration of this study.

Mibolerone Radioimmunoassay

The radioimmunoassay of the plasma samples for the presence of mibolerone was done with some expectation that an insight into the delivery rate for mibolerone would be gained. Unfortunately, mibolerone was undetectable in the plasma samples from the test females. The fact that mibolerone was undetected in the plasma is not alarming because in tests performed at the Upjohn Company it was proven that when mibolerone was mixed with food, estrus was prevented even though mibolerone was not detected in the plasma[1]. The assay sensitivity, 1.8 parts per billion, for the present study, was insufficient to detect an effective dose of the mibolerone.

Drug Diffusion Rate of the Delivery Device

Diffusion rate for mibolerone across a silastic membrane was not known at the beginning of this study. A calculated estimate for the daily dosage of mibolerone was based on the known diffusion rate for testosterone (Simmons and Hamner, 1973). The initial daily dosage of mibolerone was set to theoretically deliver 60 µg/day, which is double the recommended oral dose for preventing estrus in the

[1]Jenaay M. Brown, D.V.M., Reproduction Research, The Upjohn Company, Kalamazoo, Michigan.

beagle. The delivery devices were filled with enough of the ethanol-mibolerone mixture to deliver for approximately 5 months at 60 µg of mibolerone/day. All delivery devices were empty of ethanol when removed at 3 months, but delivery devices (3) visually inspected at the end of 10 weeks had approximately 0.1 ml of ethanol remaining. The delivery devices were disassembled and dried after removal from each animal. The devices then were visually inspected for the presence of mibolerone crystals in the drug chamber. The fact that no delivery device appeared to contain mibolerone crystals indicates that mibolerone diffusion rate exceeded 60 µg/day.

Gross Examination for Mibolerone Overdose

The delivery device may have been delivering a daily dosage greater than 60 µg of mibolerone/day, in which case some overdose effects could be expected. But clitoral enlargement, ocular and vaginal discharges, all known overdose effects, did not occur among any of the treated animals. The fact that these symptoms were not present could mean that a higher dosage than what is required by oral dose may be necessary for effectiveness by this route. It may also mean that a higher than normal dosage can be given intravaginally without eliciting an overdose response.

Estrus Resumption after Treatment

All females resumed normal estrous cycles following cessation of the intravaginal treatment with mibolerone. The time lapse following delivery device removal until the next estrus varied with each animal. Estrus was observed to recur 14 days to 238 days after treatment with mibolerone was discontinued. These results are consistent with reported observations of other investigators (Sokolowski and Geng, 1977).

Body Weight

The use of anabolic steroids has been known to cause an increased appetite with a corresponding increase in food consumption resulting in an increase in body weight. No significant ($P<0.05$) weight differences between the animals treated with mibolerone and untreated animals could be determined in the present study. But long term mibolerone treatment effects on weight gain can not be evaluated here and it is possible that weight gains may occur with prolonged treatment.

Monitoring During Treatment to Determine the Phase of the Estrous Cycle

Normally, the canine experiences estrus either during or about the same time that the plasma progesterone levels increase (Figure 13). There were alterations in the normally expected progesterone levels for proestrus, estrus and diestrus in the mibolerone treated animals. The progesterone levels of 12 mibolerone treated beagles remained at the low levels normally seen in anestrus (0.0 µg to 1.0 µg/ml of plasma) during the test period in which estrus was expected to occur. In addition, mibolerone treated females (X02, U06 and P011) which did have increases in progesterone levels during the test period did not exhibit the progesterone increases normally expected during diestrus (Figure 13). A study conducted within the Upjohn Company (Reimers, 1978) indicated that similar progesterone increases may have occurred in mibolerone treated female beagles they were testing. However, very little data were published, so complete comparisons are not possible. The most practical explanation for the observed increases in progesterone levels, for beagles X02, U06 and P011, must be based upon follicular development. Low level progesterone production begins with secondary follicular development in the ovary. It is assumed that these 3 beagles (X02, U06 and P011) underwent secondary follicular development, began producing progesterone,

but never ovulated. The anovular (atretic) follicles then began degenerating without the normal corpora lutea developing, which prevented the normal increases in plasma progesterone levels from being observed. Instead, the lower than normal plasma progesterone levels were produced from the degenerating anovular follicles.

Gross examination for vulvar swelling and vaginal discharge did not indicate proestrus or estrus for mibolerone treated animals during the treatment period. Also, the vaginal smears taken during this same period indicated an anestrus condition (Table 3). The fact that no mibolerone treated beagle demonstrated behavioral receptivity, normally seen during estrus, further indicates that "true" estrus did not occur during the treatment period.

The statistical analysis has indicated a significant ($P<0.001$) difference between the progesterone level means of the mibolerone treated and the nontreated beagles. This, then, implies that the intravaginal drug delivery device did provide an adequate daily dosage of mibolerone to prevent estrus from occurring throughout the test period.

Delivery Device Internal Pressure

The delivery device designed for this study was unique in that it used a spring to create a positive pressure (Figure 1). The main purpose for having a high internal pressure (Table 1) within the delivery device was to minimize the effect of diffusion into the drug chamber from the vaginal canal. In the present study, the osmotic and intravaginal pressures outside the silastic membrane were thought to be very small. The fact that the delivery device contained 0.8 ml of the ethanol-mibolerone solution initially and appeared completely void of all materials at the end of the treatment, suggests that the delivery device internal pressure was great enough to predominate osmotic and mechanical pressures which were encountered in the vaginal canal.

In addition, the spring tension may be adjusted to allow for some control of the ethanol-mibolerone flow rate. For example, if the membrane were thought of as a porous system and the flow of a solution through each pore were described by Poiseuille's law, then:

$$Q = \frac{N\pi R^4 \Delta P}{8\eta L}$$

Where Q = flow (in ml/min), when N = the number of pores in the membrane; R = the radius of the pore in cm; ΔP = the pressure change in mm Hg (across the membrane); η = the

viscosity of solution in poise; and, L = the thickness of the membrane in cm. For this formula it is necessary to convert units if we want the final units for Q to be in ml/min. So, if K represents the conversion of units to derive ml/min, then:

$$Q = \frac{KNR^4 \Delta P}{\eta L}, \quad \text{and} \quad K = 31413.$$

Now, using selected theoretical values, the following flow rates may be calculated:

$$Q = \frac{(31413)(1)(0.0002 \text{ cm})^4(200 \text{ mm Hg})}{(0.01 \text{ poise})(0.1 \text{ cm})} = 1 \times 10^{-5} \text{ ml/min}$$

Whereas, a ΔP of 400 mm Hg, and all other values the same, would provide:

$$Q = \frac{(31413)(1)(0.002 \text{ cm})^4(400 \text{ mm Hg})}{(0.01 \text{ poise})(0.1 \text{ cm})} = 2 \times 10^{-5} \text{ ml/min}$$

And, if ΔP was 800 mm Hg then:

$$Q = \frac{(31413)(1)(0.002 \text{ cm})^4(800 \text{ mm Hg})}{(0.01 \text{ poise})(0.1 \text{ cm})} = 4 \times 10^{-5} \text{ ml/min}$$

Therefore, theoretically, the delivery device created for this study would have delivered 30 µg of mibolerone/day if the ethanol-mibolerone solution flow rate was adjusted for 1×10^{-5} ml/min.

The previously mentioned equation was utilized to illustrate the effect of pressure as it acts on molecules crossing a membrane. The equation was designed to fit a

porous membrane and silastic membranes, strictly defined, do not have pores or openings.

Alternative Means for Dispensing Drugs

Other pressure creating means for drug dispensation may have been used in the delivery device developed for the present study. An expandable material present in both the liquid and gas form could be contained in the chamber behind the plunger of the delivery device; for example, a fluorocarbon in a liquid-vapor combination has served as a propellant in a delivery device (Blackshear, 1979). A liquid in equilibrium with its vapor phase exerts a constant vapor pressure at a given temperature, even though the volume may change. A disadvantage of the liquid-vapor pressure method is its sensitivity to temperature changes. The liquid-vapor must be injected into the pressure chamber of the delivery device under pressure and adjusted outside the animal to deliver at body temperature and pressure within the animal.

The use of a fixed charge of compressed air or other gas to create a pressure behind the plunger (Figure 1) would not provide a constant delivery rate. A volume increase in the pressure chamber behind the plunger would result in a decrease in pressure and result in changes in

the drug flow rate.

Delivery devices using an osmotic chamber saturated with a drug rely on an osmotic pressure gradient for the drug transport. The drug is transported from the osmotic chamber through a flow moderator (capillary tube) which is of a specific size to deliver a set amount of the drug when operating at a maximum flow rate. The osmotic chamber must be saturated with an excess of the drug. The author has not seen any published data that will support the fact that this type of an implantable device has been used to deliver a drug beyond a period of two weeks.

Ethanol and Mibolerone Concentration within the Delivery Device

Presently it is believed that the linear pressure of the spring on the plunger within the delivery device helps maintain a relatively constant concentration of mibolerone in ethanol. This is accomplished by the fact that as the ethanol-mibolerone solution diffuses out, the linear pressure of the spring moves the plunger forward maintaining a relatively constant ethanol and mibolerone ratio within the solution. But, in order for the constant concentration to be maintained, the diffusing volume ratio of ethanol to mibolerone had to remain constant, even though

it is probable that ethanol and mibolerone do neither diffuse bound together nor at the same rate across the silastic membrane. It is believed that the present delivery device parameters permitted the diffusing volume ratio of ethanol to mibolerone to remain rather constant throughout the treatment period. This is supported by the fact that both the ethanol and mibolerone were gone from the delivery device at 3 months and estrus was suppressed during the treatment period.

CONCLUSIONS

From this study, the following conclusions can be drawn: 1) Mibolerone delivered intravaginally did inhibit estrus in beagles; 2) the slow releasing delivery device was successful in delivering mibolerone and it was compatible with placement in the vaginal canal; and, 3) it is believed that the delivery device could provide an economical, non-surgical, reversible means of preventing estrus in the female canine.

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APPENDIX A: ASSIMILATED PLASMA LEVELS FROM ASSORTED SOURCES

The graph presented here, depicts the normally expected hormone levels for LH, progesterone and estrogen during proestrus, estrus and diestrus in the bitch.

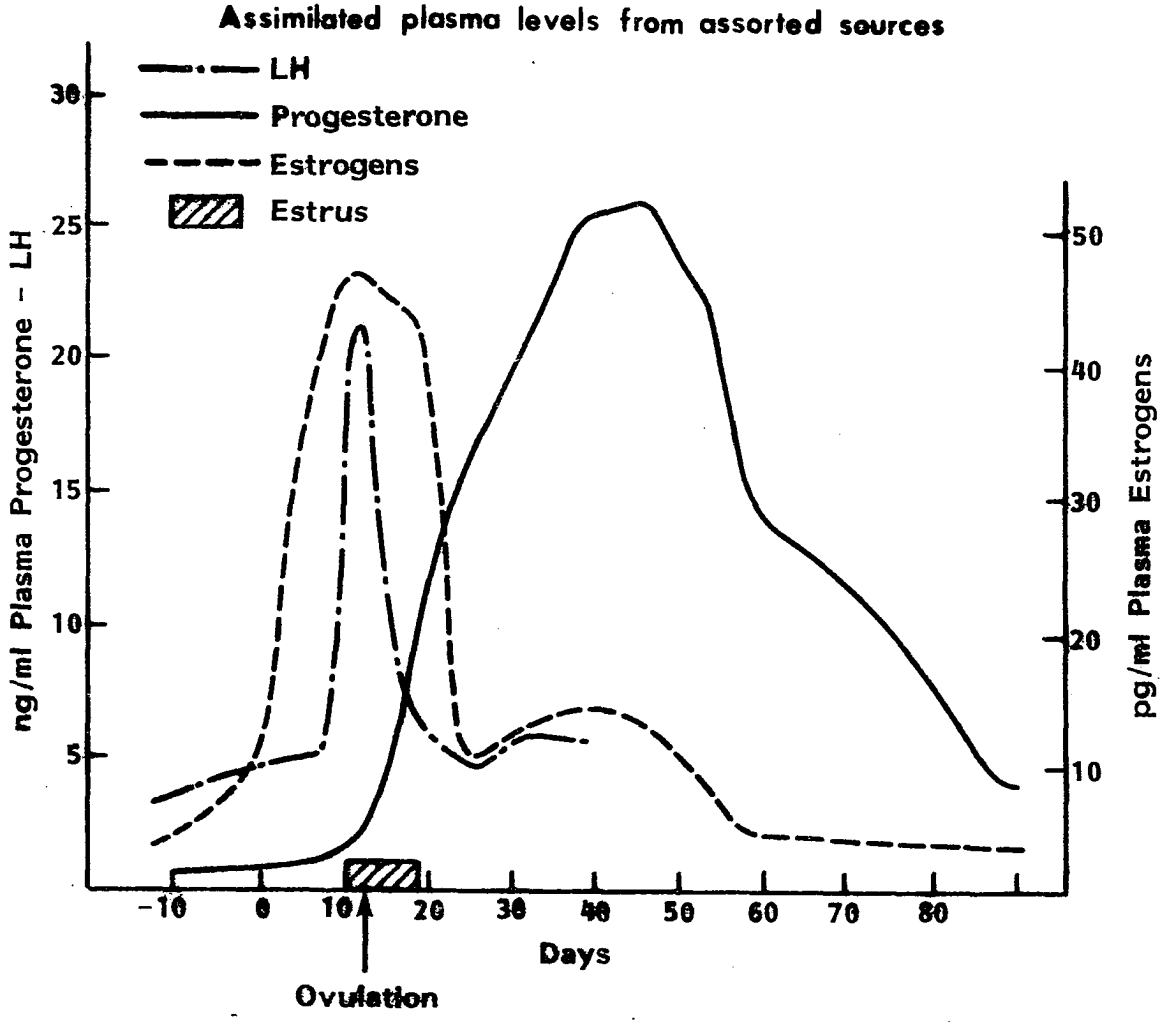

Figure A1. Normally expected hormone levels for LH, progesterone and estrogen during proestrus, estrus and diestrus in the bitch

APPENDIX B: PROGESTERONE RADIOIMMUNOASSAY

PROCEDURE

The following is a step by step procedure for the progesterone radioimmunoassay used in this study.

Extraction of the plasma:

1) Use a 16 mm by 125 mm test tube with cap.
2) Sample 100 µl of plasma (100% on the 100 µl pump) and flush with 400 µl of Tris buffer (40% on the 1 ml pump).
3) Add 4.5 ml of petroleum ether.
4) Cap tightly and shake for 15 minutes.
5) Place the test tubes in a bath of dry ice-methanol solution until the water layer freezes (or in the freezer overnight).
6) Pour off the petroleum ether phase of each sample into a 12 mm by 75 mm test tube.
7) Evaporate off the petroleum ether under air in a 40°C water bath.
8) Add 100 µl of Tris buffer to all test tubes (50% on the 200 µl pump).
9) Vortex vigorously.

The set-up of standard curve tubes:

1) Use 12 by 75 tubes.
2) With a Pipetman add:

Tube 1-Nonspecific binding (NSB)	0.5 ml Tris buffer
Tube 2-Total	0.7 ml Tris buffer
Tube 3-0 progesterone	0.1 ml Tris buffer
Tube 4-25 ng of progesterone	0.1 ml of the appropriate standard
.	"
.	"
.	"
Tube 11-3200 ng of progesterone	"

3) With the Pipetman, add 0.1 ml tracer to tubes 1 and 2.

Assay:

1) Make up the antibody in 1:25,000 dilution. Antibody is already diluted 1:1,000 and kept in the freezer in 2 ml aliquots. For one assay, add 48-50 ml of Tris buffer to the 2 ml of antibody.
2) Add antibody and the tracer simultaneously by Micromedic pipette to all tubes, excluding 1 and 2. (Antibody, 0.4 ml = 40% on the 1 ml pump and tracer, 0.1 ml = 50% on the 200 µl pump.)
3) Vortex gently and then cover with parafilm.
4) Incubate overnight in the cold room or 1 hour at room temperature and 1 hour in the cold room.

Termination of the assay:

1) Stir charcoal-dextran for about 15 minutes in the cold room before using.
2) Set timer for 15 minutes. Add 0.2 ml of the charcoal-dextran to all tubes, except the total tube (tube #2) and with the standard curve tubes in the middle of the sample tubes.
3) Vortex all tubes.
4) 15 minutes after addition of the charcoal-dextran to the first tube, centrifuge all tubes at 3000 rpm for 10 minutes.
5) Sample 0.5 ml of the assay supernatant (50% on the 1 ml pump) and flush with 4.5 ml of scintillation cocktail (90% on the 5 ml pump) into bantam vials.
6) Count on the tritium channel of the liquid scintallator for 2 or 4 minutes.

(Note - 120 test tubes could be processed at one time, the standard curve needed 11 x 2 = 22 tubes, so 98 tubes were available for samples. There were 49 samples processed in duplicate.)

The progesterone standard:

1) Weigh 10 mg P_4 and add to 10 ml methanol to make 1 mg/ml. Use a volumetric flask.
2) Dilute 1:100
   100 µl is added to 10 ml of methanol, making 10 µg/ml. Use a 100 µl Hamilton and volumetric flask.
3) Dilute 1:40
   250 µl of #2 is added to 10 ml of Tris buffer, making 250 ng/ml. Use a 500 µl Hamilton and volumetric flask.

4) Working dilutions:

250 ng/ml stock	Method	Tris buffer	P_4 conc. pg/ml	P_4 conc. in 0.1 ml
15 µl	50 µl H[1]	15 ml	250	25 pg
30 µl	50 µl H	15 ml	500	50 pg
60 µl	100 µl H	15 ml	1000	100 pg
120 µl	500 µl H	14.9 ml	2000	200 pg
240 µl	500 µl H	14.8 ml	4000	400 pg
480 µl	500 µl H	14.5 ml	8000	800 pg
960 µl	1 ml pipet.	14.0 ml	16000	1600 pg
1.92 ml	2 ml pipet.	13.1 ml	32000	3200 pg

Tracer progesterone (1,2,6, 7(n)-^{3}H progesterone):

Specific activity was 303 mCi/mg. To determine the number of pg placed in each sample tube, the following calculations were necessary:

2.2×10^6 dpm/µCi x 1000 µCi/mCi = 2.2×10^9 dpm/mCi.

2.2×10^9 dpm/mCi x 330 mCi/mg = 666×10^9 dpm/mg =

666×10^6 dpm/µg = 666×10^4 dpm/ng = 666 dpm/pg

For dilution:

1 mCi/ml to 10 ml = 0.1 mCi/ml of stock solution.

0.1 mCi/ml x 0.2 ml = 0.02 mCi; put this in 100 ml buffer.

0.02 mCi/100 ml = 2×10^{-4} mCi/ml and a 0.1 ml sample of this gives 2×10^{-5} mCi.

2.2×10^9 dpm/mCi x 2×10^{-5} mCi = 4.4×10^4 dpm or 44,000 dpm in each tube or about 20,000 cpm.

$\frac{44{,}000 \text{ dpm}}{660 \text{ dpm/pg}}$ = 66 pg in each tube.

Could try putting the 200 µl of stock in 150 ml of Tris buffer.

0.02 mCi/150 ml = 1.3×10^{-4} mCi/ml.

0.1 ml contains 1.3×10^{-5} mCi.

2.2×10^9 dpm/mCi x 1.3×10^{-5} mCi = 2.9×10^4 dpm or 29,000 dpm in each tube or about 15,000 cpm.

$\frac{29{,}000 \text{ dpm}}{660 \text{ dpm/pg}}$ = 44 pg in each tube.

[1] Hamilton flask.

Tris buffer:

0.01 M Tris
0.1% gelatin
1:10,000 Merthiolate pH 7.4

To make 2 liters:
1) 2.4228 g Tris in 1500 ml H_2O in beaker.
2) Add 2 ml merthiolate (1:10).
3) Bring to pH 7.4 with 5 N HCl.
4) Bring to 2 liter volume in a volumetric flask and then pour back into the beaker.
5) Add 2 g gelatin and stir for 30 minutes with heater at 2 or 3 until gelatin dissolves.

Merthiolate stock solution 1:10:

4 g merthiolate added to 40 ml of H_2O.

Charcoal-dextran:

1) 6.25 g charcoal added to 0.625 g dextran g dextran T-70 in 500 ml of H_2O.
2) Stir for 8 hours in the cold (a cold room).

Scintillation cocktail:

Triton-X-100	1100 ml
Toluene	2500 ml
PPO	14.5 g
POPOP	0.362 g