Prevention of Estrus in Bitches by Subcutaneous Implantation of Chlormadinone Acetate

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(Received 5 March 1992/Accepted 8 March 1993)

ABSTRACT. The efficacy and clinical safety of chlormadinone acetate (CAP) in preventing estrus were assessed in bitches on condition that CAP was subcutaneously implanted in silastic silicon rubber. To evaluate the long-term efficacy of implantation, 19 bitches were divided into 4 groups given doses of 2.5, 5, 10 and 25 mg/kg, respectively. Although estrus was observed within 13 months after implantation in all of the bitches given CAP in the 2.5 mg/kg dose, and in 13 to 15 months in 3 of the 5 bitches given the 5 mg/kg dose, it was prevented for at least 24 months in all of the bitches given doses of 10 mg/kg or more. Plasma progesterone levels remained low throughout the period of estrus prevention, indicating a close correlation with the effect of CAP. The mean body weight of the bitches in groups receiving higher doses increased slightly over the course of the experiment. Except this, no clinical, hematology or biochemistry abnormalities were found in any of the treated bitches. Another 6 bitches were given 10 to 30 mg/kg of CAP, but the implants were removed to observe the recurrence of estrus and to measure the amount of CAP in the removed implant. Estrus recurred after removal even in the bitch given 30 mg/kg. The concentrations of CAP in the plasma and the amounts of CAP remaining in the implants demonstrated the sustained release of CAP from the implants. The concentrations of CAP in the individual bitches indicated that the lowest concentration effective in preventing estrus is 0.7 *ng/ml*. Subcutaneous implantation of CAP thus proved to be safe and effective in preventing estrus in bitches for prolonged periods.—KEY WORDS: bitch, chlormadinone acetate, prevention of estrus, subcutaneous implantation.

J. Vet. Med. Sci. 55(3): 431–434, 1993

A safe and effective method of long-term birth control has long been sought for bitches. Although certain progestogens, such as medroxyprogesterone acetate [18], megestrol acetate [2, 5], norethisterone acetate [13], delmadinone acetate [9], proligestone [22] and chlormadinone acetate (CAP) [16, 17], have been known to prevent estrus in bitches when given orally or by subcutaneous injection, the long-term effects of the progestogens may produce such side effects as cystic hyperplasia of the endometrium with subsequent pyometra [1, 3, 4, 7, 10, 12, 19]. Subcutaneous implantation of a progestogen, on the other hand, has been demonstrated to have long-term efficacy in humans [8], because this method allows low, stable concentrations of the progestogen to be maintained in the plasma [6, 14]. CAP is considered one of the safer progestogens having a potent preventive effect and causing few side effects [16, 17]. We therefore adopted CAP for use with the implantation method as an effective and safe way of preventing estrus in bitches for prolonged periods. The dose-dependent efficacy of CAP and the plasma concentration effective in preventing estrus were also investigated in the present study.

MATERIALS AND METHODS

Animals: The age of all of the 25 bitches ranged from 8 to 48 months, and their body weight ranged from 6.0 to 14.0 kg at the beginning of the experiment. The estrous cycle stage was anestrus approximately 5 months after the last estrus in 21 bitches, and the beginning of proestrus in other 4 bitches. The bitches were fed a standardized dog diet (Vita-one, Nippon Pet Food, Tokyo) daily and provided with access to water *ad libitum*.

Implantation: A mixture of 20% (w/w) CAP (17α-acetoxy-6-chloro-4, 6-pregnadiene-3,20-dione) and silastic silicon rubber (MDX-4–4210 Medical Grade Elastomer, Dow Corning, Michigan), together with a coagulant for solidification, was injected into a 5 mm-diameter plastic tube to form a 30 mm-long cylindrical pellet. The pellet was cut to adjust the amount of CAP per dose. When one pellet was inadequate for a given dose, 2 or 3 pellets were used. CAP was provided by Teikoku Hormone Mfg. Co., Ltd., Tokyo.

The pellet containing CAP was implanted subcutaneously after the injection with xylazine (Celactal, Bayer, Tokyo), 2 mg/kg, intramuscularly and procaine hydrochloride (Omnicain 0.5%, Daiichi Pharmaceutical, Tokyo), 1 m*l*, intradermally in the left neck.

Experimental design

Experiment 1: Nineteen mongrel bitches were used in Experiment 1 on the long-term efficacy of CAP. The bitches were divided into the following 4 groups according to dose of CAP administered: group A (n=5), 2.5 mg/kg; group B (n=5), 5 mg/kg; group C (n=5), 10 mg/kg; and group D (n=4), 25 mg/kg. The implants were left in these bitches for 24 months after implantation. Clinical observations, plasma analyses and hematology and biochemistry studies were performed in this experiment.

Experiment 2: The implants were removed from 6 mongrel bitches in Experiment 2. Three bitches were given doses of 10, 20 and 30 mg/kg, respectively, to observe the recurrence of estrus. The implants were

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removed 12, 12 and 17 months, respectively, after implantation. The other 3 bitches were given a dose of 10 mg/kg and the implants were removed 28, 29 and 48 months, respectively. The amount of CAP remaining in the implants was determined in the 4 bitches given 10 mg/kg.

Plasma CAP and progesterone assays: Blood samples were collected from the anterior brachiocephalic vein of 2 to 4 bitches in each group 3, 6, 12, 24 and 72 hrs after implantation, and from all of 19 bitches in Experiment 1 once a month for 24 months. The samples were centrifuged at 3,000 rpm for 15 min, and preparations of plasma were frozen and stored at −20°C until assay. The concentrations of CAP in plasma were determined using a modification of the capillary-gas chromatography-mass spectrometry method described by Tsukamoto *et al.* [20]. Mean intra- and interassay coefficients of variation (CV) for the CAP assay were 3.9% and 9.0%, respectively. The threshold of detection was 10 *pg*/m*l* using 1.0 m*l* samples. The plasma was also analyzed by radioimmunoassay [21] to determine the progesterone concentration as an index of corpus luteum formation. Progesterone was assayed in 0.1 m*l* replicates with solid-phase ^{125}I RIA kits (Nippon DPC Co., Tokyo). The intraassay CV ranged between 5.8% and 8.4% and the interassay CV ranged between 6.6% and 10.0%. The threshold of detection was 50 *pg*/m*l*.

Clinical observation: Clinical observation was continued for 24 months. Body weight measurements and mammary palpation were performed once a month. The vulva was examined daily for changes.

Hematology and biochemistry studies: To determine the effects of CAP implantation on hematological values and serum biochemical constituents, blood was sampled from the anterior brachiocephalic vein of all bitches in groups A to D once a month for hematology studies and every 6 months for biochemistry studies. The following parameters were determined: erythrocyte count, hemoglobin concentration, hematocrit, leukocyte count, glutamic-oxaloacetic transaminase activity, glutamic-pyruvic transaminase activity, alkaline phosphatase activity, total protein concentration, albumin-globulin ratio, total bilirubin, blood urea nitrogen, creatinine, total cholesterol, triglyceride, phospholipid, glucose, sodium ion, chloride ion and potassium ion levels.

Amounts of CAP remaining in removed implants: After removal in Experiment 2, the external appearance of the implants was observed using a dissecting microscope. CAP was extracted from the implants with chloroform and the solvent was diluted 250-fold with ethanol. The amount of CAP remaining in the implants was measured with a spectrophotometer (UV-265, Shimadzu, Kyoto) at 285 nm.

Statistical analysis: The paired *t*-test was used for statistical analyses of the data.

RESULTS

Experiment 1

Prevention of estrus in the bitches: Estrus recurred in 8 of the 19 bitches within the 24-month observation period. In the 5 bitches given the 2.5 mg/kg dose in group A, estrus recurred 3, 5, 9, 10 or 13 months after implantation, and then continued to occur several more times before the end of the experiment. In 3 of the bitches given 5 mg/kg in group B, estrus recurred 13, 14 and 15 months, respectively, after implantation. No estrus was observed in any of the bitches in groups C and D. The implantation of CAP at the 2.5 or 5 mg/kg also suppressed the expected estrus in the 3 bitches that were at the beginning of proestrus when implanted.

Plasma concentrations of progesterone: The plasma concentration of progesterone in all of the bitches in all groups was basically stable at around 1 *ng*/m*l* or less. Although it occasionally increased up to 20 to 40 *ng*/m*l* for 1 or 2 months, this always coincided with a recurrence of estrus. There were also recurrences of estrus without any increases in progesterone concentration.

Plasma concentrations of CAP: The mean concentration of CAP in the plasma of the bitches in all of the groups rose rapidly until 12 hrs and then continued to increase gradually or kept stable until 72 hrs after implantation (Fig. 1A). The concentrations had started to decrease by 1 month after implantation and continued to decrease gradually thereafter (Fig. 1B).

Clinical signs: Vulvar inspection and mammary palpation revealed no abnormalities in any of the bitches, just normal changes associated with recurrent estrus. The mean body weight of all groups was basically stable throughout the period of the experiment, but increased slightly during the first 1 year in groups on higher doses. No abnormality was noticed in the implant site.

Hematology and biochemistry studies: Mean blood values fluctuated within the normal range in all groups and no tendencies worth noting were observed.

Experiment 2

Recurrence of estrus after implant removal: Estrus first recurred 4, 8 and 10 months after removal of the implant from 3 bitches given doses of 10, 20 and 30 mg/kg, respectively, and the second estrus at 10, 14 and 18 months, respectively. The first recurrent estrus did not result in pregnancy after spontaneous mating. Two bitches receiving 10 and 20 mg/kg, respectively, became pregnant during the second estrus and were normally delivered of 4 or 5 normal puppies which grew well, but the bitch which received the 30 mg/kg implant had become sterile.

Removed implants: Examination of the implants after removal revealed the central area of the circular cut surface to be white, but the periphery was more transparent. The longer duration of the implantation had been, the shorter the diameter of the white circle was. It was found that 58.4% of the CAP remained in the implants 12 months after implantation, and that the amount decreased thereafter to 4.9% at 48 months (Table 1).

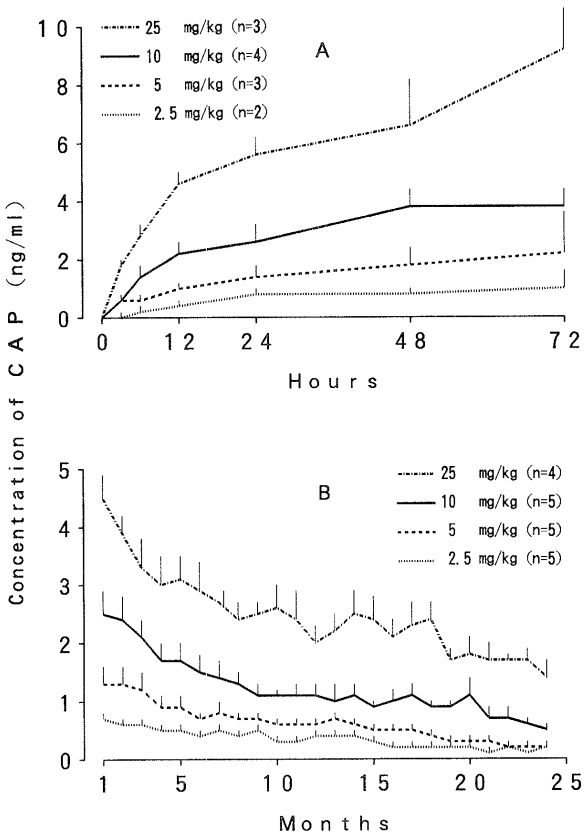

Fig. 1. Changes in mean concentrations of chlormadinone acetate (CAP) in the plasma of bitches after subcutaneous implantation of CAP. A: Changes in the concentrations immediately after implantation. B: Changes in the concentrations 1 to 24 months after implantation. The vertical bars represent standard errors.

Table 1. Amounts of chlormadinone acetate (CAP) remaining in the implants when removed at different times after implantation

Dose (mg/kg)	Duration of implantation (months)	Remaining CAP (%)
10	12	58.4
10	28	41.0
10	29	23.2
10	48	4.9

DISCUSSION

The preventive effect of a single implantation of CAP on estrus in bitches persisted dose-dependently for long periods, i.e., more than 12 months at a dose of 5 mg/kg and at least 24 months at 10 mg/kg or more. The effective dose was lower than that of norethisterone [15]. The long-term efficacy of CAP implantation could be explained by the sustained release of CAP from silicon rubber. This sustained release was confirmed by gradual decrease in amount of CAP remaining in the implants. The dose-dependently stable levels of CAP in plasma may also have resulted from gradual release, and these would explain its efficacy over long periods at high doses.

Although the exact mechanism is unknown, it has been postulated that progestogen inhibits anterior pituitary gonadotropin secretion in bitches [11]. The CAP concentration appeared to be correlated with the recurrence of estrus in bitches. The concentrations at the time of estrus were always below 0.7 *ng/ml*, and no bitches with concentrations above this level experienced recurrence of estrus. This evidence suggests that the minimum concentration of CAP effective in preventing estrus is 0.7 *ng/ml*, and that measurement of the plasma CAP concentration allows us to predict the efficacy.

Monthly measurements of plasma progesterone concentrations served as reliable evidence of the prevention of estrus by CAP. Estrus recurred in all bitches with high plasma progesterone values, however, there were also 3 bitches in which estrus recurred in the presence of low progesterone concentrations. The signs of estrus in these 3 bitches were weak and conception failed to occur, suggesting that these were anovulatory estruses.

No clinical, hematology or biochemistry abnormalities were found in any of the treated bitches, except a slight increase in body weight similar to that observed by Sawada *et al.* after oral administration [16]. The results agreed well with the observation of side effects by Sekeles *et al.* [17] who administered repeated subcutaneous injections at doses comparable to those in this study and found significantly fewer occurrences of side effects in the treated bitches than in the controls. The findings in the present study demonstrated the safety of both the implantation method itself and the administration of CAP by this method.

In conclusion, subcutaneous implantation of CAP was shown to be a safe and reliable method for preventing estrus in bitches for prolonged periods, since the level of CAP can be kept stable and removal of the implant guarantees recurrence of normal estrus, and in addition it only required a simple operation at the beginning.

ACKNOWLEDGEMENTS. The authors are indebted to Messrs. K. Tsukamoto, T. Hoshino, and H. Yorozuya, Teikoku Hormone Mfg. Co., Ltd., for determination of CAP and progesterone concentrations, and to Dr. H. Kino, Hamamatsu University School of Medicine, for his help in preparing the manuscript.

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