Pathologic Changes Related to Subcutaneous Implantation of Chlormadinone Acetate for Preventing Estrus in Bitches

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ABSTRACT. Pathologic changes related to chlormadinone acetate (CAP) implantation were examined using 14 bitches given doses 2.5 to 25 mg/kg for 2 years. Absence of corpus luteum in bitches given 5 mg/kg or more supported long-term preventive effect of CAP on estrus. The uteri were dose-dependently enlarged and mucometra was occasionally found. Endometrial epithelium hyperplasia was observed but less in smaller doses. Changes in the mammary gland were only growth and lactation at normal degree. No remarkable changes were observed in ACTH and LH cells in the pituitary gland. Low, stable levels of CAP maintained in plasma by subcutaneous implantation seemed to be the main reason for absence or slight CAP-related pathologic changes.—KEY WORDS: chlormadinone acetate, pathology, subcutaneous implantation.

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Subcutaneous implantation of chlormadinone acetate (CAP) was proved to be effective in preventing estrus in bitches for long periods [15]. This efficacy was attained by long-lasting stable levels of CAP in plasma of implanted bitches. CAP is a progestogen first synthesized by Ringold *et al.* [14] and known to possess antigonadotropic, antiestrogenic, progestagenic, antiandrogenic and glucocorticoid-like activities [7, 16]. Long-term operation of CAP at higher doses has been reported to produce various pathogenic effects in addition to estrus prevention in bitches. The pathologic changes include mammary development with subsequent mammary tumor [12, 13, 16], cystic hyperplasia of the endometrium with subsequent pyometra [9, 13], and atrophy of adrenal cortex [13, 16]. Some of these effects may limit the clinical application of this method for prevention of estrus. The long-lasting low level of CAP attained by the implantation method may cause different changes from those reported previously using oral administration. The present study examined CAP-related pathologic changes in bitches received subcutaneous implantation of CAP.

Fourteen bitches (10 to 48 months old, weighing 6.7 to 14.0 kg) were divided into 4 groups and each group received subcutaneous implantation of CAP at different doses as described in the previous report [15]: group A (n=5) given 2.5 mg/kg of CAP; group B (n=3), 5 mg/kg; group C (n=3), 10 mg/kg; group D (n=3), 25 mg/kg. In addition 2 bitches were implanted with 10 and 30 mg/kg of CAP and they were removed 12 and 17 months later, respectively, in order to examine whether the effects of CAP are reversible.

Two years after implantation of CAP, 14 bitches in groups A to D were anesthetized with sodium pentobarbital and exsanguinated via the carotid artery for pathologic examinations. After gross examination, samples from the ovary, uterus, mammary gland, adrenal gland, pituitary gland, implant site and other organs were fixed in 10% buffered formalin, embedded in paraffin, mounted and stained with hematoxylin and eosin. The additional 2 bitches given 10 and 30 mg/kg were hysterectomized at 14 and 23 months after removing implants, respectively. The uteri were similarly processed.

To examine effects of CAP implantation on the function of the anterior pituitary gland such as secretions of adrenocorticotropic hormone (ACTH) and luteinizing hormone (LH), immunohistochemical staining of the pituitary gland was performed. The specimens were sectioned at 4 μm, deparaffinized with xylene and rinsed thoroughly with ethanol. The sections were placed in absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to inactivate endogeneous peroxidase activity, and then washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 5 min each. The sections were blocked with 5% normal goat serum for 30 min and incubated for 2 hr with 1:1000 rabbit polyclonal anti-bovine LH (UCB-Bioproducts, Belgium) and 1:1000 rabbit polyclonal anti-porcine ACTH (Advance, Tokyo). The specificity of these antisera for staining of LH cells [2] and ACTH cells [5, 6] had been evaluated previously. After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed and then incubated with streptavidin-biotin complex (Histofine SAB-PO (R) Kit, Nichirei, Tokyo) for 1 hr. After washing in 0.01 M PBS, the sections were incubated in Graham-Karnovsky's reaction medium [8] which contained 3,3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka) and 0.05% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 5 to 10 min at room temperature. Then the sections were counterstained for nuclei with 1% methyl green. The number of cells per visual field of light microscope was counted at × 400. For the control, 3 non-treated Beagle bitches at anestrus were used and specimens were similarly prepared.

Histopathologic changes related to CAP implantation are summarized in Table 1. In the ovaries of the bitches

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Table 1. Histopathologic findings related to chlormadinone acetate (CAP) implantation in bitches for 2 years

Organ	Dose of CAP (mg/kg)			
Findings	2.5	5	10	25
Ovary				
Absence of corpus luteum	0	3	3	3
Uterus				
Endometrial epithelium hyperplasia				
mild	1	3	2	0
moderate	0	0	1	3
Mammary gland				
Lobular hyperplasia	2	3	3	3
Lactation	2	3	1	3
No. of bitches examined	5	3	3	3

Figures in the table represent the number of bitches that showed the changes.

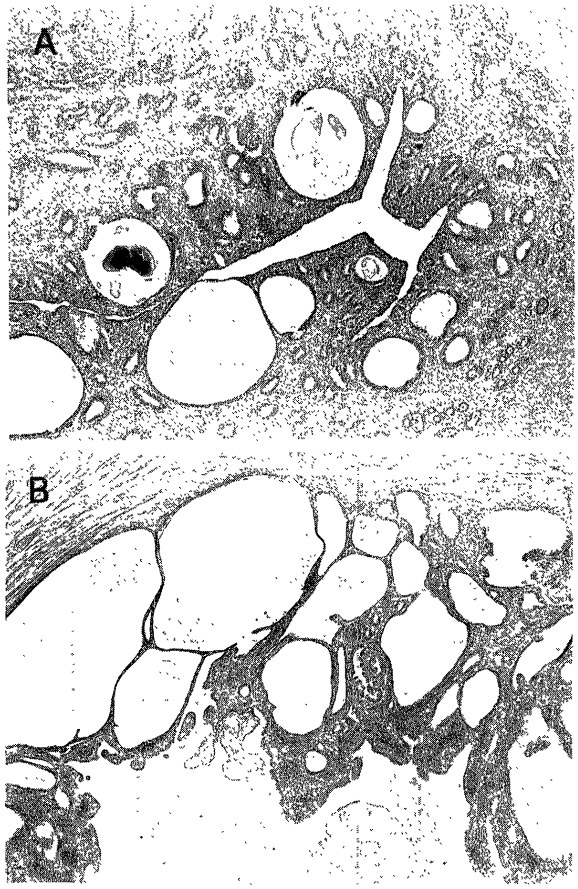

Fig. 1. Light micrograms of the uterus following treatment with 5 mg/kg (A) and 25 mg/kg (B) of chlormadinone acetate implantation for 2 years. Note different degree of endometrial epithelium hyperplasia, mild (A) and moderate (B).

given doses of 5 mg/kg or more in which estrus was prevented, developing ovarian follicles were observed but no mature follicles were observed in addition to absence of corpus luteum. These bitches also showed at gross examination dose-dependent enlargement of uteri which were occasionally found to have mucometra. In the enlarged uteri, endometrial epithelium hyperplasia was observed but less in smaller doses (Fig. 1). These changes were not remarkable in the additional 2 bitches from which implants were removed. Changes in the mammary gland were only growth and lactation at normal degree. There were no remarkable changes in the adrenal gland, implant site and other organs of all bitches. Intensity of staining and number and size of ACTH- and LH-positive cells in pituitary section of all treated bitches were not different from non-treated control and not affected by different doses (Table 2).

Table 2. Effect of CAP implantation on numbers of ACTH- and LH-positive cells in the anterior pituitary gland of bitches

Dose (mg/kg)	No. of bitches	ACTH cell	LH cell
Control	3	87.5±3.7	128.0±6.5
2.5	5	89.1±4.0	130.0±8.0
5	3	86.9±5.3	129.6±7.0
10	3	90.0±5.0	132.0±9.8
25	3	88.2±2.3	131.9±9.3

Values are expressed as mean±S.D.
Cell counts were obtained as the number of cells per visual field of light microscope at ×400. Ten fields were examined and averaged for each bitch.

The long-lasting preventive effect of CAP on estrus in bitches given 5 mg/kg or more reported in previous paper [15] was supported by absence of corpus luteum in these bitches. Presence of developing ovarian follicles and absence of mature follicles indicated that the prevention of estrus was caused by suppression of ovulation. Progestogens, when injected intra-muscularly, have been demonstrated to suppress LH surge probably through antigonadotropic activity [10]. El-Etreby and Fath-El-Bab [3, 4] showed that LH cells in the pituitary gland of Beagle bitches were atrophied after oral administration of cyproterone acetate, one of the progestogens. These observations suggested a mechanism of estrus prevention that progestogens given to bitches suppressed ovulation by suppressing LH surge due to morphological change in LH cells in the pituitary gland. In the present study, however, no morphological or numerical changes were observed by light microscopy. The evidence suggests that the morphological change in LH cells was not a substantial cause of the preventive effect of CAP on estrus, and was simply caused by a high dosage of the progestogen. Low levels of CAP by the implantation method may have suppressed LH surge only through functional changes in LH cells and/or effects on the hypothalamus. Murakoshi *et al.* [11] indicated that an anti-androgen (TZP-4238) was an androgen receptor antagonist. This suggests another possible effect of CAP to act on hormone receptor on the ovary.

The pathologic findings in the present study suggest that

CAP implantation induces very few side effects on the bitches. The proliferative changes in the endometrium would be due to progestagenic activity of CAP [1]. These changes were not remarkable in the bitches after removing implants, indicating that the reaction by CAP implantation was reversible. This would support the clinical evidence that reproductive activity recurred in bitches from which implants were removed. The presence of the proliferative changes together with absence of corpus luteum implies another mechanism of the estrus prevention that the endometrial epithelium hyperplasia in the uteri directly acted on the ovary through some substance(s) such as prostaglandin and inhibited ovulation. The proliferative changes in the mammary gland were also due to the progestagenic activity [1]. Changes in the adrenal gland and ACTH cells in pituitary section were negligible, and this indicates that the present method did not induce glucocorticoid-like activity.

Oral administration of CAP was reported to cause cystic endometrial hyperplasia with subsequent ulcerative metritis, mammary tumor and atrophy of adrenal cortex in female Beagles that were given a daily dose of 0.25 mg/kg for 2 to 4 years [13]. However, these changes were absent or slight, if at all, in the present study. The difference may have derived from different levels of plasma CAP in the animals. In bitches that received CAP by oral administration at a daily dose of 2.6 mg/kg, the plasma CAP concentration reached a stable level ranging from 144 to 291 *ng*/m*l* at the 22nd day and later (unpublished data). If the plasma concentration changes dose-dependently, the oral dose of 0.25 mg/kg/day will produce a plasma concentration of approximately 15 to 30 *ng*/m*l*. On the other hand, the subcutaneous implantation of CAP at 25 mg/kg produced plasma CAP less than 10 *ng*/m*l* at the beginning and 2 to 3 *ng*/m*l* in the period later than 5 months [15]. Thus it is clear that the subcutaneous implantation of 2.5 to 25 mg/kg of CAP in the present study produced much lower plasma concentrations than oral administration of 0.25 mg/kg/day. It was concluded, therefore, that low, stable levels of CAP maintained in plasma by subcutaneous implantation could be the main reason for few changes due to the antigonadotropic and glucocorticoid-like activities and less serious condition in the uterine and mammary glands due to progestagenic activity.

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