Tokai J Exp Clin Med., Vol. 25, No. 3 , pp.87-91, 2000

Histopathological Study of Female Beagle Dogs for Four Year Treatment with Subcutaneous Implantation of Chlormadinone Acetate (CMA)

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(Received March 16, 2000; Accepted June 20, 2000)

The histopathological changes related to chlormadinone acetate (CMA) implantation were examined using female beagle dogs given 10mg/kg for four years. All control animals showed sign of estrus during the experiment, with periods of anestrus of normal duration. In contrast, estrus was completely inhibited in the CMA-implanted animals. Histopathologically, uterine sections from the CMA-implanted animals showed cystic glandular hyperplasia, but no histologic evidence of endometritis, myometritis, and pyometra was found. In the ovaries of the CMA-implanted animals, developing ovarian follicles were observed but no mature follicles were noted in addition to an absence of corpus luteum. No remarkable changes were observed in the liver, adrenal, mammary gland, gallbladder and implanted site. Furthermore, the intensity of staining and number and size of ACTH-and LH-positive cells in the pituitary sections of CMA-implanted animals were not different from control animals. It was concluded, therefore, that subcutaneous implantation of CMA is a potential drug-delivery system for reducing changes due to antigonadotropic and glucocorticoid-like activities and characteristic histopathological changes in the uterus due to progestagenic activity.

Key words : Chlormadinone acetate (CMA), Implantation, Beagle dog, Uterus, Ovary, Oestrus cycle

INTRODUCTION

Progesterone is a steroid hormone secreted primarily by the corpus luteum. The functional activities of progesterone are related to secretory changes of the endometrial mucosa, suppression of myometrial contractions, and promotion of alveolar growth of the mammary glands [6]. Production of FSH and LH hormones by the anterior pituitary gland is inhibited by progesterone, thereby preventing follicular growth, maturation, secretion, and ovulation [6].

Larger doses or chronic oral administration of progestogens have been associated with endometritis and pyometra in bitches [1, 3, 5, 21] and queens [7, 13, 17] with diabetes mellitus or abnormal glucose tolerance tests in bitches [1, 27] and queens [7, 13, 17] with weight gains, and with mammary secretions, enlargement, and tumors [1, 20, 21] and cystic mucinous hyperplasia of the gallbladder in dogs [21]. Furthermore, some progestogens have been reported to have glucocorticoid-like activity, to cause adrenocortical suppression like glucocorticoids in rats and dogs [21].

Subcutaneous implantation of chlormadinone acetate (CMA) was proved to be effective in preventing estrus in bitches for long periods [25]. This efficacy was ascribed to the long-lasting stable levels of CMA in plasma of implanted bitches [26]. CMA is a progestogen and known to possess antigonadortopic, antiestrogenic, progestagenic, antiandrogenic and glucocorticoid-like activities [21].

The present study examined CMA-related histopathological changes in female beagle dogs receiving subcutaneous implantation of CMA for four years.

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MATERIALS AND METHODS

Animals

Fifteen female beagle dogs were purchased from Hazelton Research Product, Inc. (Denver, PA). The animals were housed individually in stainless steel cages in a semibarrier system maintained at room temperature of 22 ± 3℃, and relative humidity of 60 ± 20%, with 12 hr of light (7:00–19:00). The animals were given 250g of a standard diet (CD-1, CLEA Japan, Inc.) daily and tap water *ad libitum*. They were 1.5 to 2 years old.

Implantation

A mixture of 100 mg CMA (17alpha-acetoxy-6-chloro-4, 6-pregnadiene-3, 20-dione) and silastic silicon rubber (MDX-4-4210 Medical Grade Elastomer, Dow Corning, MI), 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 containing CMA was implanted subcutaneously after an injection with xylazine (Celactal, Bayer, Tokyo), 2 mg/kg, intramuscularly and procaine hydrochloride (Omnicain 0.5%, Daiichi Pharmaceutical, Tokyo), 1 ml, intradermally in the left neck at anestrus of the estrous cycle stage.

Experiments

The animals were divided into the following 2 groups according to dose of CMA administered: group 1 (n=5), 0 mg/kg; group 2 (n=10), 10 mg/kg. The implants were left in these animals for 2 years. They were then replaced and left for another 2 years.

Histopathological examinations

Four years after implantation of CMA, all animals were anesthetized with sodium pentobarbital and exsaguinated via the caroid artery for pathologic examinations. All of gross findings were recorded. The following organs were fixed in 10% neutral buffered formalin, embedded in paraffin following the generally accepted method and stained with hematoxylin and eosin for histopathological examination; liver, adrenal, pituitary, ovary, uterus, mammary gland, and implanted site.

Immunohistochemical staining

Formalin-fixed and paraffin-embedded sections of the pituitary glands were used. Rabbit antisera against human LH (Dako Japan, Kyoto) and human ACTH (Dako Japan, Kyoto) were used. The specificity of these antisera for staining LH-and ACTH-cells has been evaluated previously. The antisera at dilutions of 1:1000 were incubated with the sections at room temperature for 30 min. Then, the sections were covered with biotin-conjugate goat anti-rabbit IgG for 1 hr, and streptavidin-biotin-peroxidase complex (Histofine, SAB-PO (R) Kit, Nichirei, Tokyo) for 1 hr. After the incubation was completed, the sections were treated for 5 to 10 min at room temperature with Graham-Karnovsky's reaction medium [11], which contained 20 mg of 3,3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka) and 0.05% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. The sections were finally counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH 4.2. The number or cells per visual field of the light microscope was counted at × 400. Ten fields were examined and averaged for each group.

Statistical analysis

The data were expressed as mean ± S.D. Homogeneity of variance was tested by Bartlett's method, and when the assumption of homogeneity of variance was met, one-way layout analysis of variance was performed. When a significant difference was observed, Dunnett's multiple comparative test was performed between the control group and the CMA-implanted group.

RESULTS

Clinical findings

Initial mean body weights were 8.4 ± 1.2 kg (Control animals) and 8.8 ± 1.1 kg (CMA-implanted animals). At the end of administration, mean body weight of CMA-implanted animals (9.6 ± 1.5kg) was comparable to that in the control animals (9.2 ± 1.4kg). No animals died during the experimental period, and no general clinical abnormalities were observed in any of the groups. All control animals showed signs of estrus during the experiment, with periods of anestrus of normal duration. In contrast, estrus was completely inhibited in the CMA-implanted groups. There was mild to moderate mammary development in control animals for short periods following estrus, but a similar degree of enlargement in CMA-implanted animals was more persistent. No abnormali-

ty was noticed of the implanted site.

Necropsy and histopathological findings

1) Macroscopic findings

The uteri of the CMA-implanted animals were distended by the accumulation of tenacious mucoid materials. No abnormalities were noted in the other organs.

2) Microscopic findings

Histopathological findings are given in Table 1. Uterine sections from the CMA-implanted animals had moderate to severe cystic glandular hyperplasia characterized by an endometrium thickened with proliferated and dilated glands, a dilated uterine lumen, and accumulation of mucoid material. No histologic evidence of endometritis, myometritis, or pyometra was found in the uterine sections.

The ovaries from the control animals contained one or more corpora lutea. In the ovaries of the CMA-implanted animals, developing ovarian follicles were observed but no mature follicles were noted in addition to an absence of corpus luteum.

In the mammary glands of the CMA-implanted animals, lobular development with predominantly acinar proliferation and secretion occurred in various lobules. Similar changes was found for the control animals.

In both control and CMA-implanted animals, gallbladder had mild cystic mucinous hyperplasia. The hyperplastic epithelium lined numerous cystic glands filled with mucoid material. No inflammatory change or necrosis was noted.

No adrenal cortical atrophy was found in the adrenal gland sections from CMA-implanted animals. There were no remarkable changes in adrenal gland, pituitary gland, liver or implanted site of any animal.

The intensity of staining and number and size of ACTH-and LH-positive cells in pituitary sections of CMA-implanted animals were no different from control (Table 2).

DISCUSSION

The preventative effect of a single implantation of CMA on estrus in female beagle dogs persisted 24 months at a dose of 10 mg/kg. The long-term efficacy of CMA implantation could be explained by the sustained release of CMA from silicon rubber. This sustained release has been confirmed by a gradual decrease in the amount of CMA

Table 1 Histopathological findings

Organ	Findings	Untreated control	CMA(10mg/kg, four years)
		n=5	n=10
Uterus	Cystic glandular hyperplasia		
	mild	0	0
	moderate	0	7
	severe	0	3
Ovary	Absence of corpus luteum	0	10
Mammary	Acinar proliferation	5	10
Gallbladder	Cystic mucinous hyperplasia	5	10

CMA: Chlormadinone acetate

Table 2 The numbers of anterior pituitary LH- and ACTH-cells

Group	n	Dose (mg/kg)	Anterior pituitary	
			LH cells[a]	ACTH cells[a]
Untreated	5	0	139.3 ± 10.3	90.3 ± 6.0
CMA	10	10	140.5 ± 8.2	88.5 ± 3.9

Values are mean ± S.D.

[a] Cell counts are expressed as the number of cells per visual field by light microscopy at ×400.

CMA: Chlormadinone acetate

remaining in the implants [25].

The response expected from this experiment occurred mainly in the ovary, uterus, and mammary gland. The 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 intramuscularly, have been demonstrated to suppress LH surge probably through antigonadotropic activity [19]. Several authors showed that LH cells in the pituitary gland of bitches were atrophied after oral administration of cyproterone acetate [8], one of the pogestogens. These observations suggested a mechanism of estrus prevention by which 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 on immunohistochemical examination. The evidence suggests that the morphological change in LH cells, was not a substantial cause of the preventive effect of CMA on estrus, and was simply caused by a high dosage of the progestogen. Low levels of CMA may have suppressed LH surge only through functional changes in LH cells and/or effects on the hypothalamus.

Mammary lobular development occurred. There have been sporadic reports on the occurrence of mammary tumors in animals treated with progestogens [20, 21, 27]. Mammary fibroadenoma but not mammary carcinoma was found to be associated with treatment with a progestogen in a retrospective study [12]. The mechanism of action of exogenous progestogens in mammary carcinogenesis is still poorly understood. Their action may be direct or indirect. Mammary gland has been reported to have a high affinity for progesterone and receptors with low affinity for progesterone which also bing glucocorticoids [9, 10]. Some investigators believe that progesterone bound to high affinity receptors is directly responsible for stimulating mammary gland growth [4]. Such receptors have been demonstrated in mammary gland in the cat and dog, and other species [15, 24]. In addition, progesterone may act indirectly by stimulating release of certain pituitary hormones that effect growth of mammary gland. Elevated levels of GH, but not prolactin, have been reported in beagle dogs with proliferative mammary lesions, established after long-term exposure to synthetic progestins [9, 10]. The latter mechanism does not appear valid since long-term administration of megestrol acetate to animals failed to induce overproduction of pituitary GH [22]. In the present study, the proliferative changes in the mammary gland would be due to progestagenic activity of CMA. These changes were not remarkable. Changes in the adrenal gland and ACTH cells in the pituitary glands were negligible, and this indicates that the present method did not induce glucocorticoid-like activity. In this context, measurements of plasma-ACTH or cortisol levels would seem to be important to clarify this problem. Further work along this line is now in progress in our laboratory.

Cystic mucinous hyperplasia of the gallbladder mucosa, which occurred in the treated dogs, commonly occurs in aged dogs [16]. Although the drug-induced relationship was unclear, this change has been reported in dogs given progestogens [16, 18].

Uterine cystic glandular hyperplasia and pyometra have been known to be related to endocrine imbalances in bitches for many years [6, 28]. Similar changes have been produced in bitches by using the naturally occurring hormone progesterone [6]. The causal relationship of cystic glandular hyperplasia to the synthetic progestogen, medroxyprogesterone acetate, is well documented [6]. In the present study, the uteri were enlarged, but of a nearly uniform diameter throughout the length of each horn, rather than ballooned. The exudate was similar in each case: thick, translucent, non-odorous, and extremely tenacious. The microscopic lesions involving the uteri were essentially cystic endometrial hyperplasia, characterized by an unusual amount of secretion. Inflammatory changes were mild or absent and bacteria were not found. Based on our data and these facts, histopathological changes in uterus were thought to be the naturally occurring disease and the condition produced with progesterone [2, 6].

It was concluded, therefore, that subcutaneous implantation of CMA is a potential drug-delivery system for reducing changes due to antigonadotropic and glucocorticoid-like activities and characteristic histopatho-

logical changes in the uterus due to progestagenic activity.

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