

## Fertility of Bitches in which Estrus was Prevented with Implantations of Chlormadinone Acetate for Four Years

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(Received 22 January 2004/Accepted 27 September 2004)

**ABSTRACT.** The recurrence of estrus and fertility after removal of a subcutaneous chlormadinone acetate implant (CMA-I) administered to prevent estrus for 4 years, was investigated in 8 female dogs and the results compared with those for 4 untreated female dogs (control group). The sex hormones present during the estrous cycle were also investigated. There were no significant differences in the estrous cycle after removal of the implant between the CMA-I-treated group and the control group. However, although conception was achieved after mating and no uterine diseases developed in the control group, only 5 (4 dogs, 41.7%) of the 12 cases (6 dogs) in which mating took place at the second to fourth estrus after the removal of CMA-I resulted in pregnancy in the CMA-I-treated group. Furthermore, 6 (75.0%) of the 8 dogs in the CMA-I-treated group developed uterine diseases including pyometra or hydrometra. There were no significant differences in plasma progesterone, LH and prolactin levels between the non-pregnant and pregnant dogs in the CMA-I-treated group or control group. These results suggest that long-term implantation of CMA-I affects fertility after the implant is removed.

**KEY WORDS:** canine, chlormadinone acetate, fertility, implantation, pyometra.

*J. Vet. Med. Sci.* 67(2): 151–156, 2005

Many studies have investigated the use of reversible contraception procedures with synthetic hormone agents to replace permanent surgical sterilization [7, 13–15, 17]. With respect to the prevention of estrus in dogs using a synthetic progestogen, chlormadinone acetate (CMA), Sawada *et al.* [14] and Tamada *et al.* [16] have reported oral administration of CMA, and Sahara *et al.* [12, 13] have reported a procedure with a CMA implant (CMA-I). According to these studies, 2 mg/head of an oral preparation administered once a week and 10 mg/kg or more of the implant preparation administered every 2 years prevented estrus, with no clinically significant side effects during the administration period. In Japan, a CMA implant preparation was approved as an estrus-preventing agent for dogs in 1992, and has been used in clinical practice ever since. It has been reported that contraceptive synthetic progestogens prevent estrus by inhibiting superior hormone secretion; however, megestrol acetate and medroxyprogesterone acetate cause side effects in the uterus and mammary glands [2–4, 18]. Tamada *et al.* [16] also achieved contraception through oral administration of CMA for 2 to 9.8 years, and reported that mammary gland tumors were detected in 7 of 14 dogs, although no uterine disease developed during follow-up. CMA-I is more advantageous than the oral preparation in that a low blood level of CMA is maintained, minimizing progestogenic action-related systemic effects. To achieve prolonged contraceptive effects, we implanted CMA-I for 4 years, and found that this agent prevented estrus without clinically significant side effects during the implantation period; however, accumulation of mucoid materials and a moderate to severe cystic glandular hyperplasia in the uterus were induced [8]. We did not investigate fertility after low con-

centrations of hormone were given for a long period. Fertility after a 1-year implantation of CMA-I was confirmed in 2 dogs [13].

In this study, to examine the influence of long-term prevention of estrus by CMA-I on subsequent fertility, we investigated the state of recurrence of estrus and fertility after removal of CMA-I in female dogs in which CMA-I had been implanted for 4 years.

### MATERIALS AND METHODS

**Animals:** We used 12 beagle bitches ranging from 5.5 to 6 years in age and from 8 to 11 kg in body weight. In 8 of these dogs, 10 mg/kg of CMA-I (GS implant<sup>®</sup>, Teikoku Hormone Mfg. Co., Ltd., Tokyo) was subcutaneously implanted at 1.5 to 2 years of age, and an additional implantation was performed 2 years after the initial implantation to achieve contraception for 4 years (CMA-I-treated group) [8]. In 4 dogs, a pellet alone was simultaneously implanted as a placebo (control group). Furthermore, 6 male beagle dogs aged 1 to 6 years in which fecundity was confirmed were used for mating for a fertility test involving these dogs.

The animals were kept in 160 × 75 × 65 cm cages at a density of several dogs per cage. Commercial dog food (Hill's Canine Maintenance, USA) was given once daily and drinking water was given three times daily (morning, afternoon and evening). Pregnant dogs were given food twice (morning and evening) from 35 days of gestation. This study was conducted in conformity with the animal study guidelines of Nippon Veterinary and Animal Science University.

**Observation of estrus:** The female dogs were observed

every day for pudendal enlargement and the presence or absence of vulval bleeding.

**Observation of the uterus:** The uterine status in the bitches was investigated using an ultrasonic imaging diagnosis system (ECHOVISION SSD-500EV, Aloka Co. Japan) at 1-month intervals. When abnormalities in the uterus were observed, the uterus was frequently investigated. Concerning uterine diseases, pyometra was differentiated from hydrometra based on clinical symptoms such as anorexia, the state of drainage from the vulva, and the leukocyte count.

**Estimation of ovulation day and mating:** Mating was performed at estrus, 2 (3 bitches) or 3 (1 bitch) times after removal of the pellet in the control group, and 2 (5 bitches), 3 (5 bitches), or 4 (2 bitches) times after removal of CMA-I in the CMA-I-treated group. As we previously reported [5], mating was performed during the optimum mating period, 2–5 days after ovulation, based on the ovulation day estimated from the plasma progesterone ( $P_4$ ) level. Blood was collected from the anterior brachiocephalic vein daily starting 6 days after vulval bleeding at proestrus. The day on which the  $P_4$  level initially reached 2 ng/ml or higher was regarded as the ovulation day. In the control group, mating was not performed after pregnancy was observed once.

**Measurement of plasma sex hormone levels:** To investigate the kinetics of sex hormones during the estrous cycle and gestation period (complete reproductive cycle) in the bitches, blood (4.5 ml) was collected through the anterior brachiocephalic vein at 3-day intervals from the estimated date of ovulation until day 72 in non-pregnant dogs, and until 3 days after delivery in pregnant dogs. Blood samples were immediately centrifuged in a low temperature centrifuge (3,000 rpm, 20 min) to isolate plasma, and plasma samples were stored at  $-40^\circ\text{C}$  until hormone levels were measured. We measured the levels of LH and prolactin (PRL), which may be related to  $P_4$  and luteal maintenance in dogs.

Sex hormone levels were measured in the 4 pregnant dogs (4 cases) and 3 non-pregnant dogs (3 cases, Bitches 5, 7 and 10) in the control group as well as in the 3 pregnant dogs (3 cases, 21, 24 and 25), 2 non-pregnant dogs (2 cases, 21 and 24), and 2 dogs with abortion (2 cases, 16 and 25) in the CMA-I-treated group. In the CMA-I-treated group, these levels were not measured in dogs with uterine diseases.

Plasma  $P_4$  was measured by an enzyme immunoassay method developed by Munro and Stabenfeldt [9]. The intra-assay coefficient of variation for samples was 7.2%, and the inter-assay coefficient of variation for the same pools was 11.4%. The sensitivity of this immunoassay method was 0.25 pg/well.

Plasma LH was measured using a double-antibody radioimmunoassay (RIA) method in accordance with the procedure described by Nett *et al.* [11], except that radiolabelled porcine LH (LER-778) and anti-porcine LH serum were used, as reported by the authors [6]. Purified canine LH (LER-1685) was used as the standard. The assay standard

curve was done in duplicate, using 10 standard concentrations ranging from 0.098 to 50 ng/ml. Samples were assayed in duplicate using 100  $\mu\text{l}$  aliquots. The intra-assay and inter-assay coefficients of variation were 10.8% and 7.7%, respectively. The minimum detectable concentration was 0.20 ng/ml.

Plasma PRL levels were determined by a homologous RIA method [1]. RIA determinations were made using a double antibody method. Highly purified cPRL (AFP-2451B; a gift from Dr. A.F. Parlow, Pituitary Center, UCLA, Los Angeles, CA) was used for iodination and for the standard curve. The assay standard curve was done in duplicate, using 10 standard concentrations ranging from 0.098 to 50 ng/ml. Samples were assayed in duplicate using 100  $\mu\text{l}$  aliquots. The intra-assay coefficients of variation for samples with high and low levels were 10.4 and 8.2%, respectively. The inter-assay coefficients of variation for samples with high and low levels were 8.5 and 7.9%, respectively. The minimum detectable concentration of canine PRL was 0.20 ng/ml.

**Diagnosis of pregnancy:** Pregnancy was determined 25 days after ovulation using an ultrasonic imaging diagnosis system. Pregnant bitches were observed every five days after the determination of pregnancy to confirm normal embryonic development until delivery. The newborns were enumerated on the delivery days.

**Statistical analyses:** Data obtained in this study were analyzed by Student's *t*-test and a significance level lower than 5% was defined as significant.

## RESULTS

The state of recurrence in the estrous cycle after removal of CMA-I and the results of conception are shown in Table 1. In the control group, the first estrus after removal of the pellet was expressed as the period (days) since the previous estrus.

The interval between the removal of CMA-I and the initial estrus was a mean of  $205.6 \pm 86.0$  (SD) days. Concerning the subsequent reproductive cycle, the mean estrous cycle and the complete reproductive cycle showed no marked differences between the CMA-I-treated group and the control group.

During the anestrus following the second estrus after removal of CMA-I, 2 dogs (Bitches 19 and 22) developed hydrometra, and in each case the uterus was removed. At the third estrus, the 5 mated dogs were all sterile. Three dogs (18, 23 and 25) developed pyometra. In 2 dogs, 18 and 25, *Enterobacter cloacae* and *Enterococcus faecium* were detected on bacterial examination, respectively. Of the 3 dogs, an ovariohysterectomy was performed in one dog (Bitch 18), a  $\text{PGF}_{2\alpha}$ -analogue (50  $\mu\text{g}$ /head of fenprostalene, Dai-Nippon Pharmaceutical Co., Ltd.) and an antibiotic were administered to another (23), and drainage and uterine lavage were performed by laparotomy in the other dog (25). Treatment was successful in all 3 cases. At the fourth estrus, 3 of 5 mated dogs became pregnant. However, the 2 dogs

Table 1. The state of recurrence of the estrous cycle after removal of CMA-I and the results of conception in the CMA-I-treated group

Bitch Number	Days from removal to estrus	Number of recurring estrus	Reproductive cycle (day)		Gestation period (days from ovulation)	Presence or absence of uterine disease (bacterium)	Number of pups		
			Incomplete	Complete					
CMA-I treated group	16	165	1	186	a)	— <sup>b)</sup>	Pyometra <sup>c)</sup>	Abortion, 29 (33)	
			2	227					
			3	214					
			4	213					
	18	244	1	197			Pyometra		
			2	140		—	( <i>Enterobacter cloacae</i> )		
	19	204	1	261			Hydrometra (—) <sup>d)</sup>		
	21	182	1	239					
			2	167		—			
			3	227		59 (64)		4	
			4	245					
	22	401	1	293			Hydrometra (—)		
	23	141	1	392					
			2	170		—	Pyometra <sup>c)</sup>		
			3	329		—	Pyometra <sup>c)</sup>		
	24	169	1	266					
			2	247		—			
			3	274		64 (66)		9	
			1	212					
	25	139	2	181		—	Pyometra		
			3	188		62 (64)	( <i>Enterococcus faecium</i> )	1	
			4		260	Abortion, 26 (30)			
Mean ± SD	205.6 86.0		238.4 <sup>e)</sup> 55.9		61.7 (64.7) 2.5 (1.1)		4.7 4.0		
Control group	5	—	1	225					
			2	255		62 (64)		6	
			3						
	7	—	1	224					
			2	201					
			3	134		60 (65)		5	
			4		217				
			5	159					
	9	—	1	415					
			2	341		62 (64)		8	
	10	—	1	201					
			2	245		60 (62)		8	
			3		258				
			4	176					
Mean ± SD			234.2 72.2		61.0 (63.8) 1.5 (1.7)		6.8 1.0		

a) Mating b) Sterile c) Not bacterial examination d) Asepsis e) Excluding 3 cycles shortened after administration of PGF<sub>2α</sub>-analogue (Bitch Nos. 16–4, 23–3, 25–3)

(16 and 23) that did not become pregnant at this time developed pyometra 18 and 21 days after ovulation, respectively, and ovariohysterectomy was performed in 1 dog (Bitch 23), while a PGF<sub>2α</sub>-analogue (50 µg/head of cloprostenol,

Teikoku Hormone Mfg. Co., Ltd.) and an antibiotic were administered to the other dog (16). Treatment was successful in both cases. At the fifth estrus, 2 mated dogs (16 and 25) became pregnant; however, abortion occurred.

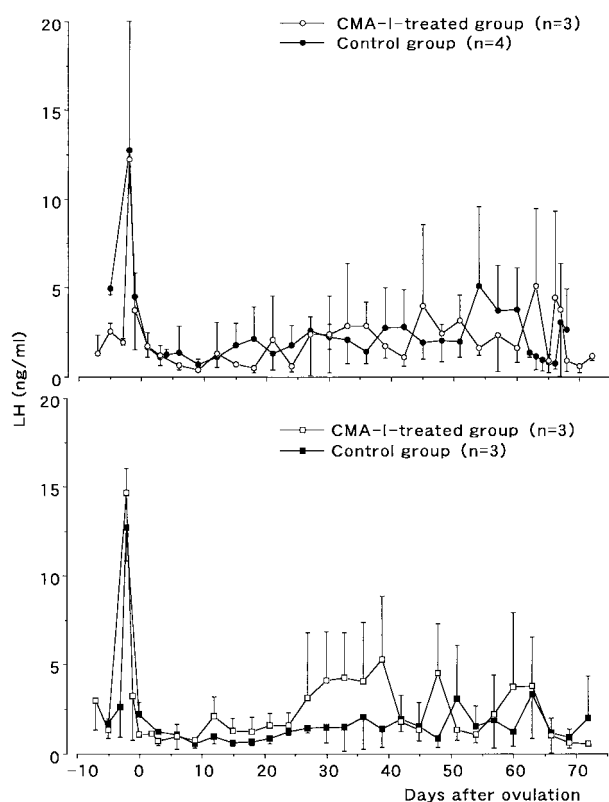


Fig. 1. Changes in the plasma LH levels (Mean  $\pm$  SD) in the pregnant (upper) and non-pregnant (lower) dogs in the CMA-I-treated and control groups.

In the control group, conception was achieved in all mated dogs, and no dog developed any uterine disease during the reproductive cycle.

Plasma LH, PRL and  $P_4$  levels in the pregnant and non-pregnant dogs in the CMA-I-treated and control groups are shown in Fig. 1–3.

Among the pregnant dogs, changes in the plasma level of LH were similar between the two groups, whereas among the non-pregnant dogs, the plasma level of LH was slightly higher in the CMA-I-treated group, although the difference was not significant.

In the two groups, the plasma level of PRL in the pregnant dogs increased gradually 30 or more days after ovulation. The value was lower in the CMA-I-treated group, although not significantly. In both groups, the plasma level of PRL was low in the non-pregnant dogs.

Among both pregnant and non-pregnant dogs, the plasma level of  $P_4$  was slightly lower in the CMA-I-treated group.

Plasma LH, PRL, and  $P_4$  levels in the 2 dogs with aborted fetuses in the CMA-I-treated group are shown in Fig. 4.

Changes in the plasma levels of LH and  $P_4$  were similar to those during the pregnancy/non-pregnancy periods in the control group. Furthermore, changes in the plasma level of

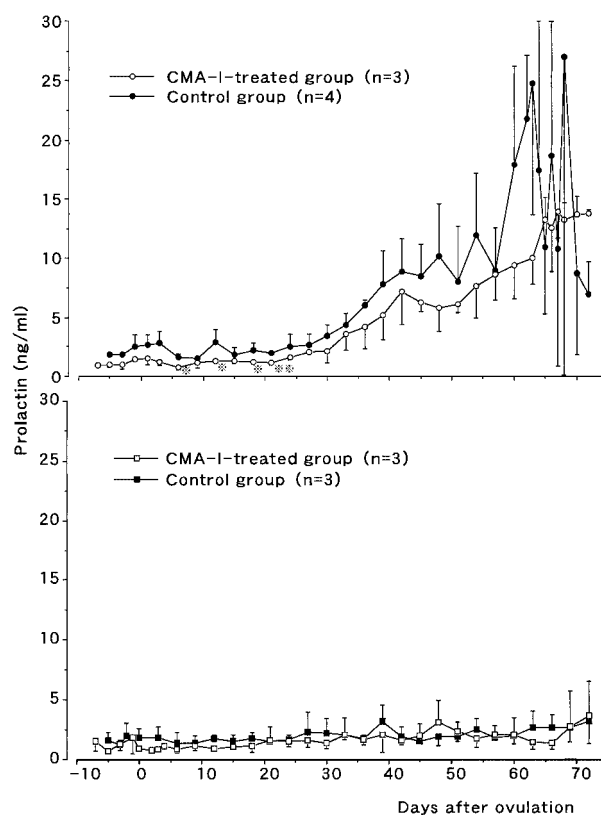


Fig. 2. Changes in the plasma prolactin levels (Mean  $\pm$  SD) in the pregnant (upper) and non-pregnant (lower) dogs in the CMA-I-treated and control groups. Significantly different from the control groups at  $p < 0.05$  (\*),  $p < 0.01$  (\*\*).

PRL were similar to those during the non-pregnancy period in the control group, although the value was slightly higher in one dog (Bitch 16).

## DISCUSSION

We implanted CMA-I in beagles for 4 years. This preparation prevented estrus without serious side effects, although the accumulation of mucoid materials and a moderate to severe cystic glandular hyperplasia in the uterus were induced [8]. However, 6 (75.0%) of the 8 dogs in the CMA-I-treated group developed uterine diseases after the removal of CMA-I.

Inhibition of the secretion of pituitary hormones for a long period or the direct influence of CMA on the uterus may have been involved in these diseases. However, there were no changes in the interval between removal of the CMA implant and the initial estrus or subsequent estrous cycles, and our previous study [8] confirmed that atrophy of the pituitary gland did not occur. In this study, there were no significant differences in plasma sex hormone levels after the removal of CMA-I between the CMA-I-treated group and control group, suggesting that changes in sex hor-

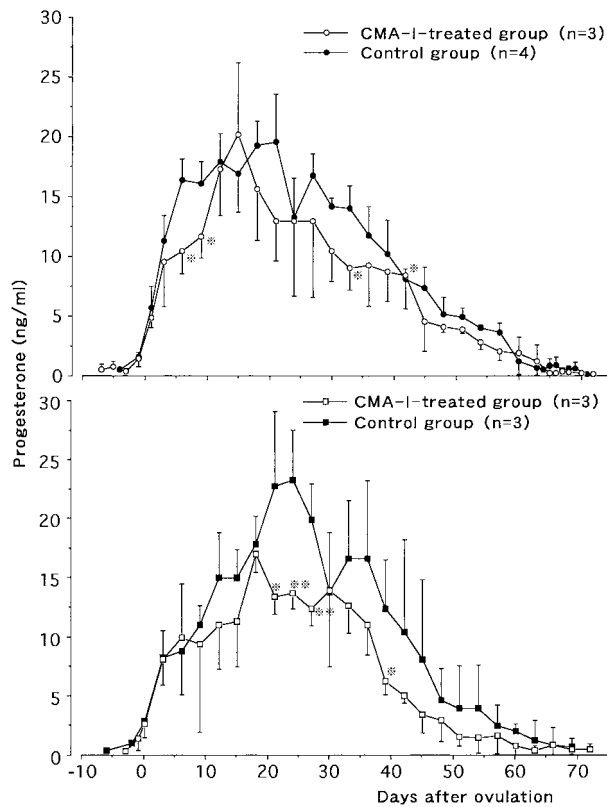


Fig. 3. Changes in the plasma progesterone levels (Mean  $\pm$  SD) in the pregnant (upper) and non-pregnant (lower) dogs in the CMA-I-treated and control groups. Significantly different from the control groups at  $P < 0.05$  (\*),  $p < 0.01$  (\*\*).

more levels are not involved in sterility or a high incidence of uterine diseases.

The pathogenesis of pyometra remains to be clarified in some respects; however, this disorder is known to develop during the luteal phase in old nulliparous female dogs [9, 10]. All dogs used in this study were nulliparous dogs in which prevention of estrus had been performed since 1 to 2 years of age, and this fertility test was performed at 7 to 8 years of age. No uterine disease developed in the untreated group, therefore, the influence of CMA on the uterus over a long period may have led to an environment of cystic endometrial hyperplasia, which readily causes bacterial proliferation, a factor involved in uterine diseases. This may have contributed to the low conception rate and the high incidence of uterine diseases seen in the present study. The influence of repeated normal cycle-related sensitization with estrogen and  $P_4$  on the uterus after long-term implantation of CMA and bacterial invasion from the cervical canal during estrus may have contributed to the onset of uterine disease.

To investigate the recurrence of estrus after removal of the CMA-I, we did not perform mating at the first estrus after the removal. However, there were no abnormalities in

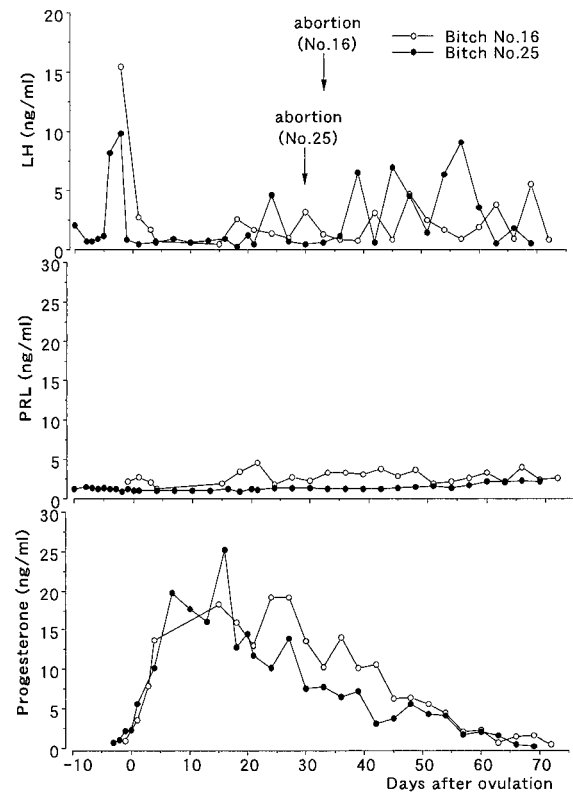


Fig. 4. Changes in the plasma LH, prolactin, and progesterone levels in the 2 dogs (Bitches No. 16 and 25) with abortion in the CMA-I-treated group.

the uterus until the second estrus. Therefore, the rate of conception when mating is performed early after removal should be investigated.

These results suggest that long-term implantation of CMA-I affects fertility after removal of the implant. On the basis of this result we recommend that CMA-I only be used for short-term administration, a period of 2 years or less.

**ACKNOWLEDGEMENT.** The authors are indebted to Masashi Tagawa, Rie Ikeda and Toshio Iwasaka, Teikoku Hormone Mfg. Co., Ltd. for their kind cooperation.

#### REFERENCES

1. Concannon, P.W. 1993. Biology of gonadotrophin secretion in adult and prepubertal female dogs. *J. Reprod. Fertil. (Suppl.)* **47**: 3-27.
2. Brodey, R.S. and Fidler, I.J. 1966. Clinical and pathological findings in bitches treated with progestational compounds. *J. Am. Vet. Med. Assoc.* **149**: 1406-1414.
3. Dow, C. 1957. The cystic hyperplasia-pyometra complex in the bitch. *Vet. Res.* **69**: 1409-1415.
4. Frank, D.W., Kirton, K.T. and Murchison, T.E. 1979. Mammary tumors and serum hormone in the bitches treated with

- medroxyprogesterone acetate or progesterone for four years. *Fertil. Steril.* **31**: 340–346.
5. Hase, M., Hori, T., Kawakami, E. and Tsutsui, T. 2000. Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. *J. Vet. Med. Sci.* **62**: 243–248.
  6. Kawakami, E., Tsutsui, T. and Ogasa, A. 1990. Peripheral plasma levels of LH, testosterone, and estradiol-17 beta before and after orchiopey in unilaterally cryptorchid dogs. *Jpn. J. Vet. Sci.* **52**: 179–181.
  7. McCann, J.P., Altszuler, N., Hampshire, J. and Concannon, P.W. 1987. Growth hormone, insulin, glucose, cortisol, luteinizing hormone, and diabetes in beagle bitches treated with medroxyprogesterone acetate. *Acta Endocrinol.* **116**: 73–80.
  8. Murakoshi, M., Ikeda, R., Tagawa, M., Iwasaka, T. and Nakayama, T. 2001. Histopathological and immunohistochemical studies in female beagle dogs during four years treatment with implantation of chlormadinone acetate (CMA). *J. Toxicol. Pathol.* **14**: 9–12.
  9. Munro, C. and Stabenfeldt, G.H. 1984. Development of a microtiter plate enzyme immunoassay for the determination of the progesterone. *J. Endocrinol.* **101**: 41–49.
  10. Nelson, R.W., Feldman, E.C. and Stabenfeldt, G.H. 1982. Treatment of canine pyometra and endometritis with prostaglandin F<sub>2α</sub>. *J. Am. Vet. Med. Assoc.* **181**: 899–903.
  11. Nett, T.M., Akbar, A.M., Phemister, R.D., Holst, P.A., Reichert, L. E. Jr. and Niswender, G.D. 1975. Levels of luteinizing hormone, estradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch. *Proc. Soc. Exp. Biol. Med.* **148**: 134–139.
  12. Sahara, K., Murakoshi, M., Nishina, T., Kino, H. and Tsutsui, T. 1994. Pathologic changes related to subcutaneous implantation of chlormadinone acetate for preventing estrus in bitches. *J. Vet. Med. Sci.* **56**: 425–427.
  13. Sahara, K., Tsutsui, S., Naitoh, Y. and Fujikura, K. 1993. Prevention of estrus in bitches by subcutaneous implantation of chlormadinone acetate. *J. Vet. Med. Sci.* **55**: 431–434.
  14. Sawada, T., Tamada, H., Inaba, T. and Mori, J. 1992. Prevention of estrus in the bitch with chlormadinone acetate administered orally. *J. Vet. Med. Sci.* **54**: 595–596.
  15. Sekeles, E., Lange, A.D., Samuel, L. and Aharon, D.C. 1982. Oestrus control in bitches with chlormadinone acetate. *J. Small Anim. Pract.* **23**: 151–158.
  16. Tamada, H., Kawate, N., Inaba, T. and Sawada, T. 2003. Long-term prevention of estrus in the bitch and queen using chlormadinone acetate. *Can. Vet. J.* **44**: 416–417.
  17. Van Os, J.L. and Oldenkamp, E.P. 1987. Oestrus control in bitches with proligestone, a new progestational steroid. *J. Small Anim. Pract.* **19**: 521–529.
  18. Weikel, J.H., Nelson, L.W. and Reno, F.E. 1975. A four-year evaluation of the chorionic toxicity of megestrol acetate in dogs. *Toxicol. Appl. Pharmacol.* **33**: 414–426.