THE EFFECTS OF CHLORMADINONE ACETATE (CMA), ANTIANDROGEN, ON THE PITUITARY, TESTIS, PROSTATE AND ADRENAL GLAND OF THE DOG WITH SPONTANEOUS BENIGN PROSTATIC HYPERPLASIA

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ABSTRACT The effect of chlormadinone acetate (CMA), a synthetic steroidal antiandrogen, on spontaneous benign prostatic hyperplasia (BPH) in dogs was investigated. Male beagle dogs (5-8 years old) were divided into four experimental groups. Group 1 consisted of untreated controls. Groups 2 and 3 received CMA 0.03 and 0.1 mg/kg/day, p.o., respectively, for 6 months. In group 1, glandular hyperplasia of the prostate was clearly detected. The glandular epithelial cells showed uniformly intense nuclear staining for androgen receptor (AR). AR was also localized in the nuclei of the fibro-muscular stromal cells. In groups 2 and 3, CMA produced marked atrophy of the glandular epithelium. The interacinar fibro-muscular stroma was prominent. The nuclear staining for AR in both epithelial and stromal cells was remarkably decreased. In addition, a histopathological study showed that CMA medication for 6 months exerted no effect on the testes and adrenal glands or on immunoreactive positive cells to LH- and ACTH-antibody (pituitary LH- and ACTH-cells). Therefore, it is concluded that CMA (0.03 and 0.1 mg/kg) causes regression of spontaneous canine BPH without any histopathological effects on the testes, adrenal glands or pituitary LH- and ACTH-cells.

KEY WORDS: Benign prostatic hyperplasia (BPH), Beagle dog, Androgen receptor (AR), Chlormadinone acetate (CMA), LH cells, ACTH cells

INTRODUCTION

Among laboratory animals, the dog is the only species that spontaneously develops benign prostatic hyperplasia (BPH) with a high frequency (Walsh and Wilson, 1976; DeKlerk *et al.*, 1979). Canine BPH is believed by many investigators to be an appropriate model for the study of human BPH, although there are important differences between the conditions in the two species (Geller *et al.*, 1975; Okada *et al.*, 1988; Tunn *et al.*, 1988; Forti *et al.*, 1989). The human disease is often a multinodular process thought to arise in a peri-urethral stromal nodule, which is then secondarily invaded by glandular elements (Harada *et al.*, 1994). In contrast, canine BPH is a diffuse epithelial or glandular process with less stromal involvement (DeKlerk *et al.*,

1979). Although some differences exist between human and canine BPH, the dog is considered to be a good animal model of BPH to test the efficacy of drugs that cause shrinkage of the hyperplastic gland (Geller *et al.*, 1975; Okada *et al.*, 1988; Tunn *et al.*, 1988; Forti *et al.*, 1989).

Chlormadinone acetate (CMA)(Fig. 1) is a steroidal antiandrogen that is widely used in the medical management of BPH in Japan (Ito *et al.*, 1977; Harada *et al.*, 1994). It is a well-documented fact that CMA inhibits the uptake of testosterone in the prostate and is selectively incorporated into prostate cells, resulting in inhibiting testosterone binding to the cytosol 5 α -dihydrotestosterone (DHT)-receptor (Ito *et al.*, 1977; Takezawa *et al.*, 1992,1995).

Previously, the atrophic effect of a synthetic

steroidal antiandrogen, CMA, on spontaneous BPH in dogs was investigated (Murakoshi *et al.*, 2000). As a results, CMA produced marked atrophy of the glandular epithelium and the interacinar fibro-muscular stroma was prominent. Furthermore, the intensity of the immunostaining for androgen receptor (AR), 5 alpha-reductase type I and type II was negative or very weak under CMA treatment. Therefore, it is concluded that atrophy after treatment with CMA may be due to shrinkage of both glandular and stromal compartments in the prostate tissue (Murakoshi *et al.*, 2000).

In humans, BPH is the result of an increase in both glandular and stromal compartments (Wilson, 1980). Therefore, it is suggested that effects on both compartments are required to achieve the intended clinical benefits in patients treated with CMA.

On the other hand, the atrophic effects (glucocorticoid-like activity) of cyproterone acetate (CPA) and CMA on the adrenal glands of rats have been reported by several authors (Brennan and Kraay, 1963; Starka *et al.*, 1972). Furthermore, it is well known that the above steroidal antiandrogens inhibit gonadotropin (LH) secretion and testosterone biosynthesis, unlike non-steroidal antiandrogens, which produce increases in LH and testosterone when given alone (Namer, 1988).

The purpose of the present study was to further examine the effect of CMA on canine spontaneous BPH. The effects of CMA on testicular, adrenal morphology, pituitary LH- and ACTH-cells were also investigated.

MATERIALS AND METHODS

Animals

Thirty male beagle dogs were purchased from

Fig. 1. Chemical structure of chlormadinone acetate (CMA).

Hazelton Research Product, Inc. (Denver, PA). The animals were housed individually in stainless steel cages at a room temperature of $22 \pm 3^\circ\text{C}$, and relative humidity of $60 \pm 20\%$, with 12 hr of light (7:00-19:00). The animals were given 300 g of a standard diet (CD-1, CLEA Japan, Inc.) daily and tap water *ad libitum*. They were 5-8 years old and contained BPH on the basis of biopsy (data not shown).

Experiments

Four animals served as BPH untreated controls (group 1). Groups 2 and 3 were administered orally 0.03 (group 2, $n=4$) and 0.1 (group 3, $n=4$) mg/kg/day of CMA as a crystalline powder in gelatin capsules for 6 months. All animals were sacrificed by exsanguination under pentobarbital anesthesia at the end of the experimental period.

Organ weight

The weights of the prostates, pituitary glands, testes and adrenal glands were recorded (absolute weight). Weights relative to body weight (relative weight) were calculated.

Histopathological examination

The prostates, pituitary glands, testes and adrenal glands were removed immediately, fixed in 0.1 M phosphate-buffered 10% formalin and embedded in paraffin. Cut sections were mounted and stained with hematoxylin and eosin (HE).

Immunohistochemical staining

1. Androgen receptor (AR)

The prostates were frozen in dry-ice-cooled ethanol. Frozen sections ($6\mu\text{m}$ in thickness) were prepared in a cryostat and mounted on glass slides. The sections were fixed for 10 min at 4°C in Zamboni's fixative (Zamboni and McMartino, 1967). After washing in 0.01 M phosphate-buffered saline (PBS) containing 20% sucrose, the sections were soaked in absolute methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to inactivate endogenous peroxidase. After washing in 0.01 M PBS, the sections were incubated overnight at 4°C with NH 27, a rabbit polyclonal antiandrogen receptor antibody (1:1000). After washing in 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed in 0.01 M PBS and then treated with streptavidin-biotin-peroxidase complex (Histofine, SAB-PO (R) Kit, Nichirei, Tokyo) for 1 hr. After incubation was completed, the sections were treated for 5 to 10 min at

room temperature with Graham-Karnovsky's reaction medium (Graham and Karnovsky, 1966), 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.

2. LH- and ACTH-cells

Formalin fixed and paraffin sections were used. Rabbit antisera against bovine LH (UCB-Bioproducts, Belgium) and porcine ACTH (Advance, Tokyo) were used. The specificity of these antisera for staining LH- and ACTH-cells have been evaluated previously (El Etreby and Fath El, 1977). The antisera at 1:1000 dilution were incubated with the sections at room temperature for 30 min. Then, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 hr, washed in 0.01 M PBS and then treated with streptavidin-biotin-peroxidase complex (Histofine, SAB-PO (R) Kit, Nichirei, Tokyo) for 1 hr. After incubation was completed, the immunoperoxidase staining was performed as described above. The number of cells per visual field of light microscope was counted at $\times 400$. Ten fields were examined and averaged for each group.

Statistical analysis

The data were expressed as mean $\pm$ SD. Homogeneity of variance was tested by Bartlett's method (Yoshimura, 1997), and when the assumption of homogeneity of variance was met, one-way layout analysis of variance was performed. When a significant difference was observed, Scheff's or Dunnett's multiple comparative test (Yoshimura, 1997) was performed between the control group and the other experimental groups.

RESULTS

Organ weight

As shown in Table 1, absolute and relative weights of the prostates in group 3, and absolute weights of the prostates in group 2 were statistically significantly ($p < 0.05$) reduced, compared to the control values. In contrast, pituitary, testicular and adrenal weights showed no significant differences among the groups (Table 1).

Light microscopic findings

1. Prostates

In group 1, glandular epithelial cells were markedly hypertrophic and showed an increased number of papillary projections extending into the acini (Photo 1A). Thus, histological features of glandular hypertrophy and/or hyperplasia were evident (Table 2). The amount of interacinar stroma was variable but not extensive. The glandular epithelial cells showed uniformly intense nuclear immunostaining for AR (Photo 2A). AR was also localized in the nuclei of the fibromuscular stromal cells.

In CMA-treated animals (groups 2 and 3), the glandular epithelial cells were markedly atrophic and the acini completely atrophic (Photo 1B). Thus, histological features of glandular atrophy were evident. In contrast, the interacinar fibro-muscular stroma was prominent (Table 2). By CMA-administration, intensity of the immunoreaction for AR was dose-dependently decreased in both glandular epithelial cells and fibromuscular stromal cells (Photo 2B).

2. Testes

No evidence of abnormal spermatogenesis was seen in the seminiferous tubules. No changes in the Leydig cell population were found. No evidence of Leydig cell hyperplasia or atrophy was observed in any of the experimental groups.

3. Adrenal glands

No conspicuous changes were noted in any of the experimental groups.

4. Pituitary LH- and ACTH-cells

LH cells were located mainly in the dorsal region of the pars distalis. Administration of CMA produced no significant treatment-related changes in the number of immunoreactive cells (Table 1). ACTH cells were located in both pars distalis and pars intermedia. Administration of CMA produced no significant treatment-related changes in the number of immunoreactive cells (Table 1).

DISCUSSION

In the present study, CMA produced marked atrophy of the glandular epithelium. In addition, loss of secretory and metabolic activities was evident. These findings were in good agreement with previous findings (Murakoshi *et al.*, 2000). It is a well-documented fact that CMA inhibits the uptake of testosterone in the

Table 1. Effect of CMA on prostatic weight, adrenal weight, testicular weight, pituitary weight, pituitary LH and ACTH cells.

Group	n	Dose (mg/kg)	Prostatic weight (g)		Adrenal weight (g)		Testicular weight (g)		Pituitary weight (mg)		Anterior pituitary	
			Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	LH cells ^b	ACTH cells ^b
1	4	0	25.62 ± 1.35	1.54 ± 0.35	1.25 ± 0.18	0.13 ± 0.01	15.78 ± 1.27	1.35 ± 0.22	76.56 ± 3.52	6.23 ± 1.85	138.5 ± 9.2	88.2 ± 4.5
2	4	0.03	14.36 ± 1.94 ^a	1.12 ± 0.56	1.35 ± 0.21	0.14 ± 0.05	15.92 ± 1.39	1.50 ± 0.28	76.33 ± 4.01	6.30 ± 1.96	134.6 ± 10.0	89.8 ± 5.5
3	4	0.1	9.38 ± 1.50 ^a	0.88 ± 0.31 ^a	1.30 ± 0.18	0.13 ± 0.04	16.23 ± 0.85	1.42 ± 0.31	75.98 ± 3.85	5.89 ± 0.98	140.9 ± 8.8	90.3 ± 4.2

Values are mean ± S.D.

^a: p<0.05, significant difference from BPH control (Group 1).^b: Cell counts are expressed as the number of cells per visual field by light microscopy at ×400.

prostate and is selectively incorporated into prostate cells, resulting in inhibiting testosterone binding to the cytosol 5 alpha-DHT-receptor (Ito *et al.*, 1977; Takezawa *et al.*, 1992, 1995). Thus, the uptake of testosterone and/or its androgenic effect on the prostate may be suppressed by CMA. In fact, immunostaining of nuclear AR in both epithelial and fibro-muscular stromal cells was markedly decreased after treatment with CMA. It is further well-documented that prostatic nuclear AR contents were decreased after treatment with gonadotropin-releasing hormone (GnRH) agonist (Forti *et al.*, 1989) as well as CPA (Huang *et al.*, 1985), an antiandrogenic agent. Based on our present data and these facts, decreased immunostaining of AR after treatment with CMA may be explained by a decrease in the number of AR. In fact, CMA inhibits the binding to androgen and AR competitively (data not shown). Therefore, CMA binds competitively to the AR from the prostate, and dose-dependent oral administration causes regression of the hyperplastic prostatic weight.

Table 2. Histopathological findings.

Organ	Findings	CMA (mg/kg)		
		0 (n=4)	0.03 (n=4)	0.1 (n=4)
Prostate	Glandular hypertrophy/hyperplasia	4	0	0
	Glandular atrophy			
	Mild	0	3	2
	Moderate	0	1	1
	Severe	0	0	1
	Prominence of fibro-muscular stroma			
	Mild	0	3	2
	Moderate	0	1	1
	Severe	0	0	1
	Decreased number of AR-positive cells			
	Mild	0	3	2
	Moderate	0	1	1
	Severe	0	0	1
Testis	Atrophy of seminiferous tubules			
	Mild	0	0	0
	Moderate	0	0	0
	Severe	0	0	0
Adrenal	Atrophy of cortex			
	Mild	0	0	0
	Moderate	0	0	0
	Severe	0	0	0
Pituitary	Decreased number of LH cells	0	0	0
	Decreased number of ACTH cells	0	0	0

The AR is a member of a steroid superfamily of ligand-inducible intracellular regulators that activate or repress transcription of target genes (Chang and Kokonitis, 1988). ARs are structurally and functionally organized into domains that mediate hormone binding, nuclear translocation, dimerization, DNA binding, and transcriptional activation (Beato, 1989; Carson-Jurica *et al.*, 1990). It is, therefore, conceivable the CMA might present receptor dimerization, might increase AR turnover, and thus reduce AR content. Further studies are needed to clarify the way in which CMA reduces AR.

It is well known that steroidal antiandrogens such as CPA inhibit gonadotropin (LH) secretion and testosterone biosynthesis (Namer, 1988). The present histopathological study showed that CMA (0.03 and 0.1 mg/kg) medication for 6 months exerted no effect on the testes or pituitary LH cells. Therefore, it is suggested that CMA in smaller doses (0.1 mg/kg or less) causes regression of spontaneous canine BPH without any significant histopathological changes in the testes or pituitary LH cells.

It is generally accepted that CMA has some glucocorticoid-like activities in rodents, and suppression of adrenal function is evident in rodents (Brennan and Kraay, 1963; Givner and Rochefort, 1972). The present study showed that CMA (0.03 and 0.1 mg/kg) medication for 6 months exerted no effect on the adrenal glands or pituitary ACTH cells. Therefore, it is suggested that CMA in smaller doses (0.1 mg/kg or less) causes regression of spontaneous BPH without any significant histopathological changes in the adrenal glands or pituitary ACTH cells.

AR has been observed exclusively in the nuclei of the adrenal cortical cells (Murakoshi *et al.*, 1994). The male and female adrenal showed the same pattern of AR staining. It is believed that adrenal androgens are secreted by zona reticularis. Thus, AR in the adrenal cortical cells may be involved in an autocrine function. Previously we found that AR was also localized in the nuclei of the rat adrenal cortical cells of the atrophic cortical zones induced by CMA treatment (Murakoshi *et al.*, 1994). Pham-Huu-Tung *et al.* (1984) reported that CPA, an antiandrogen, decreased cortisol production and increased adrenal androgen levels. Androgens have been shown to increase both synthesis (Blondeau *et al.*, 1982; Syms *et al.*, 1985) and half-life of AR (Syms *et al.*, 1985), which result in elevated receptor protein levels within the tissues. Therefore, it is suggested that rat adrenal cortical cells of the atrophic cortical zones induced by CMA treatment were decreased

Photo 1. A: Prostate of a dog with spontaneous BPH. Glandular hyperplasia is dominant. HE, $\times 150$.
B: Prostate of a dog with spontaneous BPH after treatment with CMA 0.1 mg/kg/day. The glandular epithelium is atrophic, and interacinar stroma is prominent. HE, $\times 250$.

Effect of CMA on canine spontaneous BPH.

Photo 2. A: Immunolocalization of androgen receptor (AR) in prostate of a dog with spontaneous BPH. The glandular epithelial cells show uniformly intense immunostaining for AR. AR is also localized in the nuclei of the fibro-muscular stromal cells (arrow). Peroxidase-labeled antibody method, $\times 150$.
B: Prostate of a dog with spontaneous BPH after treatment with CMA 0.1 mg/kg/day. The immunoreaction for AR is negative or very weak in both glandular epithelial cells and fibro-muscular stromal cells. Peroxidase-labeled antibody method, $\times 250$.

in cortisol and/or corticosterone production and increased or remained in adrenal androgen production. The biological significance of AR in the adrenal cortical cells remains to be further clarified.

It is concluded that CMA (0.03 and 0.1 mg/kg) causes regression of spontaneous canine BPH without any histopathological effects on the testes, adrenals or anterior pituitary LH-and ACTH-cells.

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