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Study of the Effect of an Anti-Androgen (Oxendolone) on Experimentally Induced Canine Prostatic Hyperplasia *

II. Endocrinological Analysis

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Summary. The results of hormonal investigation of effects of an antiandrogen, Oxendolone (OXD), alone or in combination with medroxyprogesterone acetate (MPA), on experimentally induced canine BPH are reported. Seventeen beagle dogs were divided into 5 groups: 2 castrate controls for Group 0, 3 BPH controls for Group 1 which received 3α-androstanediol (3α-A) and estradiol (E_2) for one year, and 12 dogs consisting of 3 groups which received 3α-A and E_2 for 6 months followed by testosterone propionate (TP) and E_2 for another 6 months. The last 3 groups were treated with either 200 mg/week of OXD (Group 3) or OXD + 30 mg/week of MPA (Group 4), and otherwise untreated with these hormones (Gourp 2, T-E controls). On the blood hormone analysis, both T and 5α-dihydrotestosterone (DHT) were generally lowered in the T-E administered groups (Group 2, 3 and 4) compared to those in Group 1. While MPA significantly descreased these androgen levels, OXD did not influence at all. The concentrations of E_2 were similar. Although T content in the prostate did not differ significantly within the experimental groups, DHT was the highest in Group 1 and the lowest in Group 4 and was nearly the same level to Group 0. MPA reduced tissue DHT content but OXD did not. In the receptor assay study, nuclear androgen receptor (AR) content in Group 3 and 4 was significantly lower than that in Group 1. No obvious decrease in nuclear AR content could be seen among the other groups. Significant differences were not observed in both the cytosol AR contents and in the cytosol and nuclear estrogen receptor (ER) contents, although they were generally low in Groups 3 and 4.

Key words: Canine BPH – Experimentally induced – Androgen content – Plasma estrogen – Androgen receptor – Estrogen receptor

* Nomenclature and abbreviations of hormones used in this paper are indicated in Part I

Introduction

In the previous part of this report, we demonstrated the morphological effect of Oxendolone (OXD) on volume and morphology of canine BPH experimentally induced by the Walsh and Wilson method [21]. OXD is a potent antiandrogen which acts mainly by competition at the receptor of androgen sensitive target tissues. The substance was administered alone or in combination with medroxyprogesterone acetate (MPA).

In order to carry out the experiments in a not too advanced stage of BPH, and also with histological features similar to those in the human, testosterone propionate (TP) and estradiol (E_2) were used throughout the experiment after the induction of BPH by 3α-androstanediol (3α-A) and E_2 during the first 6 months. OXD alone induced a reduction in size and atrophic changes in the BPH histology. The effect was not very pronounced and not statiscally significant. These findings were markedly enhanced when OXD was used in combination with an anti-estrogenic steroid, MPA which in large dose is also effective as an anti-androgen.

In this study the results obtained on the endocrinological aspects are documented. It mainly deals with the amounts of androgens and the receptor levels for androgen and estrogen in canine BPH.

Table 1. Experimental scheme of induction and hormonal treatment of BPH in dogs

Induction of BPH	
3α-androstanediol (3α-A)	75 mg/week
estradiol (E_2)	0.75 mg/week
Hormone manipulation	
0: Castrated control (sesame oil 1 ml/w + aq. vehicle 2 ml/w)	
1: BPH control (3α-A 25 mg/w + E_2 0.25 mg/w)	
2: T-E control (TP 10 mg/w + E_2 5 μg/w)	
3: T-E + anti-androgen (Oxendolone 200 mg/w)	
4: T-E + OXD + anti-estrogen (MPA 30 mg/w)	

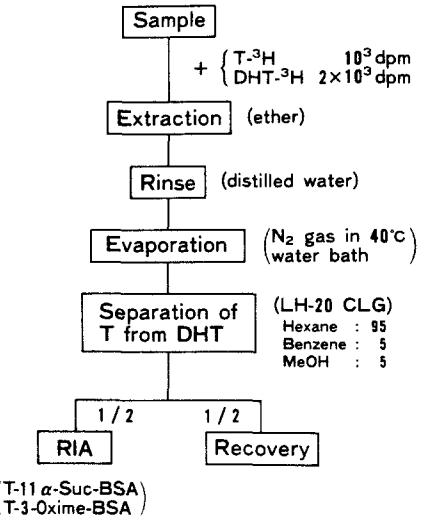

Fig. 1. Outline of the procedures to determine androgen content in the blood and the prostate. Pretreatment for the extraction of hormones from the tissue fragments is required prior to determining tissue level

Materials and Methods

The outline of the experimental protocol is shown in Table 1.

Measurement of Hormone Levels[1]

The androgen content in the prostate, and the androgen and estrogen concentration in the blood were determined in the samples obtained at the end of the experiment. Testosterone (T), 5α-dihydrotestosterone (DHT) and E_2 were measured by RIA by the methods of Makino et al. [11], Hosaka [6], and Kambegawa et al. [8], respectively.

Hormones were extracted with ether directly from blood plasma or from a tissue homogenate which had been prepared in 5 vol of saline, and then fractionated with Sephadex LH-20 column chromatography after evaporating the ether zone. These fractions were then incubated with 1,000 dpm of tritiated T*, DHT*, or E_2* and their anti-sera in co-existence with 0.25 ml of buffer solution for 20 min. Either DCC solution (RIA buffer solution containing 0.25% charcol Novit A and 0.025% Dextran T-70) for T or 50% of ammonium sulfate for DHT and E_2 was then added. The specific readioactivity in the supernatant of the centrifugates (1,500 x g, 10 min) of the mixture was finally measured (Fig. 1).

Determination of OXD Concentration

OXD in the blood was measured by RIA, using a portion of ethanol-resuspended extract of sera with ether which had been evaporated. After evaporation, it was incubated in 0.5 ml of a RIA buffer (40 mM phosphate buffer solution containing 2 mM EDTA and 0.1% BSA, pH 7.4) containing 10,000 dpm of ^{3}H-OXD* and anti-OXD-antisera [19], for 30 min at 37 °C and for 2 h at 0 °C. RIA buffer, containing 0.5 ml of DCC, was added, and the mixture was kept in an ice bath for 10 min after vortex. The radioactivity was then measured in 0.5 ml of supernatant of the centrifugate (1,500 x g, 5 min).

OXD in the tissue was assayed by HPLC, by using a Nucleosil column for 30 min of flow time. From the tissue homogenate in 4 vol fo 1% $NaHCO_3$, the OXD was extracted with n-hexane, and then eluted in methanol-water (13:7) at the flow rate of 0.5 ml/min. The OXD concentration was measured by spectrophotometry at a wave length of 254 nm. The assay limit of OXD was 0.1 ng/ml for RIA and 5 ng/g in the HPLC.

Androgen and Estrogen Receptor Assay in the Prostate

Preparation of Cytosol Fraction (CF) and Nuclear Extract (NE). Approximately 0.8 g of the prostate was homogenized in 5 ml of a PEG buffer solution using a glass-glass homogenizer. After centrifugation in 800 x g for 10 min, it was divided into supernatnat and precipitate. The former was re-centrifuged in 105,000 x g for 60 min, the supernatant of which was used as a CF after dilution with a PEG buffer to total 10 ml. PEG buffer solution is 5 mM phosphate buffer containing 1 mM EDTA and 10% glycerol, adjusted at pH 7.4. The precipitate was incubated with 5 ml of TEK buffer solution at 0 °C for 30 min, and was re-centrifuged in 5,000 x g for 10 min. The supernatnat thus obtained served as the NE. TEK buffer solution is composed of 10 mM Tris-HCl buffer solution containing 1.5 mM EDTA, 0.6 M KCl and 10 mM Na_2MoO_4, adjusted at pH 7.4.

Measurement of Protein and DNA. Cytosol and nuclear protein was determined by Bradford's method [1] by using a Bio-Rad protein assay kit purchased from Bio Rad Laboratories, Lichmond, CA. After dilution of 10 μg of the cytosol and NE fraction above obtained with 10 times of saline, the light absorption at 595 nm wave length was measured by spectrophotometry in co-existence with 5 ml of the refined reactive agent. The standard curve was prepared by the kit accompanying the BSA.

DNA was measured by the fluorescence technique using the Hoechst 33258 agent [9], obtained form Wako Pure Chemical Reagent Co., Osaka. It was determined by measuring the fluorescence at 458 nm wave length evoked at 356 nm, 30 min after dissolving 10–50 μg of the tested samples in 2 ml of a 50 mM phosphate buffer solution containing 2 M Nacl and 5 μg of Hoechst 33258.

Receptor Assay. The androgen and estrogen receptor (AR and ER) assay was done by the Garola and McGuire method [5]. A hydroxyapatite (HAP, Bio-Rad Laboratoreis, Lichmond, CA) suspension was firstly prepared by adding 0.7 vol of a HAP buffer to the precipitate after irrigation with the buffer of the precipitate by centrifugation at 1,500 x g for 3 min. 0.15 ml of the suspension was transferred into a plastic tube coated with 0.1% BSA and centrifugated by 1,500 x g for 3 min. Into this precipitate, 0.1 ml of the cytosol fraction and the same volume of a PEG buffer solution were given. The cytosol receptor was then absorbed to HAP by vortex and kept in an ice bath at 0 °C for 30 min. After this procedure it was again centrifuged at 1,500 x g for 3 min, and the supernatant was evacuated. To the precipitate, 4 μl of ethylalcohol containing 200,00 dpm of ^{3}H-DHT or the same count of ^{3}H-E_2 and 20 μl of 100 mM Na_2MoO_4 were added. They were then incubated overnight at 0 °C after diluting to 0.2 ml with a PEG buffer solution (5 mM buffer containing 1 mM EDTA and 10% glycerol, pH 7.4), containing 1.25 mM dithiothreitol (DTT). For non-specific binding, 20 μl

[1] The labeled steroid hormones which appear in the text* were obtained as follows: {6,7-^{3}H(N)}-Oxdendolone (^{3}H-OXD, 30 Ci/m mol) and {1,2-^{3}H(N)}-testosterone (^{3}H-T, 250 Ci/mol) was purchased from Amersham International plc, Buckinghamshire, England, and {1,2,4,5,6,7-^{3}H(N)}-5α-dihydrotestosterone (^{3}H-DHT, 123 Ci/m mol) and {2,4,6,7-^{3}H(N)}-estradiol (^{3}H-E_2, 115 Ci/mol) were obtained from New England Nuclear Co., Boston, MA, USA

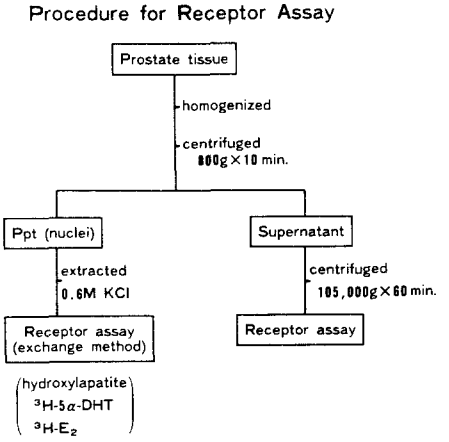

Fig. 2. Outline of the procedure of the receptor assay

of 1% EtOH-TE solution containing 4 μM of DHT or E_2 was further added to the former precipitate. After incubation, the mixture was rinsed with 1.5 ml of a HAP buffer solution three times, containing 1% Twin 80 for the ER assay. The precipitate was transferred into the vial, and 0.6 ml of EtOH was added. The radioacitivty was then measured with an ACS II scintilation counter.

Nuclear ER was assayed in the same manner by using 0.2 ml of NE, as was mentioned above, except for the following points: incubation was done at 30 °C for 2 h after adding ^{3}H-ER and rinsing with a HAP buffer solution; after being kept at 0 °C for 15 min, the nuclear AR was also measured in the NE. 0.2 ml of NE was transferred into a plastic tube and was incubated togehter with 40 μl of 1 mM Mersaryl at 0 °C for 30 min. Then 60 μl of 100 mM DTT was added. After vortex it was incubated at 0 °C for 30 min after adding 0.15 ml of HAP suspension. After centrifugation in 1,500 x g for 3 min, ^{3}H-DHT of 300,000 dpm/6 μl EtOH and 20 μl of 100 mM Na_2MoO_4 were added to the precipitate. 30 μl of 4μM DHT/1% EtOH-TE was further added to the above, and it was diluted to 0.2 ml with a PEG buffer and was used for nonspecific binding. These were kept at 0 °C overnight, and the specific radioactivity was determined in this precipitate.

The steps of the receptor assay were outlined in the figure (Fig. 2).

Results

The results obtained were summarized into 3 categories as follows:

1) measurement of hormones in the blood and in the prostate,

2) measurement of the amount of protein and DNA for receptor determination,

3) receptor assay of androgen and estrogen in the tissue.

Hormonal Examination

Hormone Concentration in Blood. The serum T and DHT levels were highest in Group 1, 677 ± 18 pg/ml and 1,767 ± 149 pg/ml (mean ± SE), and lowest in Group 4, 229 ± 76 pg/ml and 91 ± 25 pg/ml (mean ± SE), respectively. OXD administration did not reduce but tended to increase the androgen level when compared to the T-E controls, though the difference was not significant. These phenomena were partly due to inexplicably low DHT level in 2 of the 4 dogs. In the 2 castrated dogs the androgen levels were indetectable.

The serum E_2 concentrations were nearly the same in groups 2–4 but slightly lower in Groups 0 and 1 (Table 2).

Hormone Content in the Prostate. The androgen, T and DHT, content in the tissue correlated well with that in the blood (Table 2). However, the extremely high concentration of DHT in Group 1 compared to the T concentration resulted from the 3α-A administration. Similarly the disproportionally low DHT in the Group 4 prostates seemed to be the effect of MPA, as Lax et al. reported [10]. They demonstrated that systemic administration of MPA decreased in 5α-reductase activity and increased in 3α- and 3β-hydrocysteroid dehydrogenase activity in the liver cells of castrated rats.

Irrespective of the type of treatment, the DHT content per gram of the prostate correlated well with the size (r =

Table 2. Estrogen and androgen concentrations in the blood and adrogen content in the prostate

Group	blood (pg/ml)			prostate (ng/g)	
	E_2	T	DHT	T	DHT
0: castrated control	38.7 ± 3.1	< 100	< 20	0.61 ± 0.07	0.12 ± 0.02
1: BPH control	40.0 ± 1.5	677 ± 18 [a]	1,769 ± 149 [b]	0.92 ± 0.03	5.72 ± 0.24 [a]
2: T-E control	49.8 ± 1.2	360 ± 67 [a]	364 ± 301 [b]	0.62 ± 0.08	2.41 ± 1.05 [a]
3: T-E + OXD	52.1 ± 3.2	451 ± 93 [b]	606 ± 200 [b]	0.78 ± 0.10	3.01 ± 1.16 [b]
4: T-E + OXD + MPA	47.7 ± 3.7	229 ± 76 [b]	91 ± 25 [b]	0.59 ± 0.09	0.46 ± 0.22 [b]

mean ± SEM
[a] significantly different at $P < 0.05$
[b] $P < 0.01$

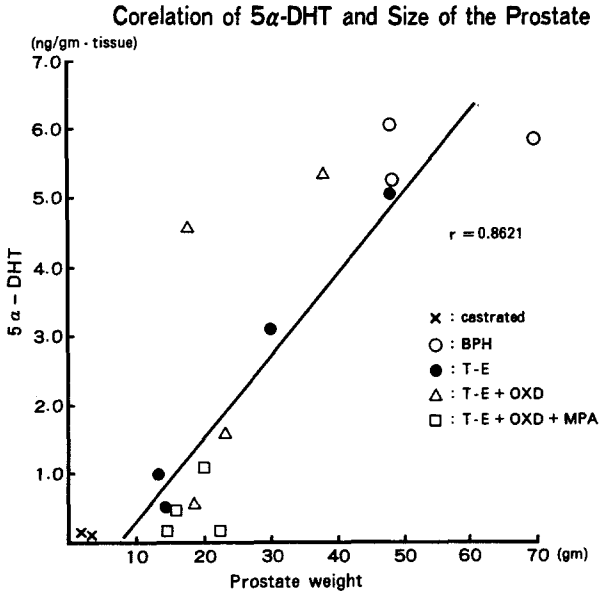

Fig. 3. Correlation between dihydrotestosterone (DHT) contents in the prostate gland and the weight of the gland. Close correlation is noticed between two parameters (r = 0.86)

Table 3. Oxendolone (OXD) level in the blood and in the prostate of the OXD-treated dogs

Group	Dog No.	Serum (ng/ml)	Prostate (ng/g)
3: T-E + OXD	A 482	39.0	41.0
	B 282	46.9	78.0
	B 582	52.1	50.2
	W 182	34.6	63.6
		43.2 ± 3.9	58.2 ± 8.1
4: T-E + OXD + MPA	A 582	40.5	51.4
	B 182	60.6	44.9
	B 482	48.8	81.2
	H 582	34.8	65.0
		46.2 ± 5.6	60.6 ± 8.0

0.86), as indicated in Fig. 3 which had been suggested by Moore et al. [13]. (Fig. 3). These findings also correlated well with the histological findings (Part I. of this report), i.e. the typical glandular BPH in Group 1 and the severe atrophic change in Group 4.

OXD Concentration. The OXD distributions in the blood and prostate tissue were almost at the same level in Groups 3 and 4, which implies that MPA did not affect the OXD distribution in the prostate. The concentration of OXD was slightly higher per gram tissue than per ml of serum. This is shown in Table 3.

Protein and DNA Content

The protein content in the cytosol (CF) and the nucleus (NE) in the entire prostate as well as per gram of tissue have been summarized in Table 4 where DNA in the NE is indicated in the same manner. These data were more correctly expressed by cell number (Table 4).

Receptor Assay

The AR and ER contents were expressed per DNA content in the tissue, since the values given by protein unit were widely scattered. Nuclear AR, ER, and cytosol ER were highest in Group 1, followed by Groups 2, 3, and 4 in this order (Figs. 4, 5).

The AR in Groups 3 and 4 when expressed x 10^3 dpm/mg DNA were significantly reduced compared to Group 1, $P < 0.05$ and 0.01, respectively. However, the differences between Groups 2, 3, and 4 were not significant. Taking the relatively low DHT tissue concentration in Groups 3 and 4 into consideration, OXD seem to have exerted its antiandrogenic activity by competing at the receptor level. The AR content was the lowest in Group 0.

While ER, both in cytosol and nucleus, was present in amounts similar to those of AR, no significant change could be observed among the groups, except for the castrate control.

Table 4. Amount of cyostol and nuclear protein and DNA in the nuclei in each experimental

Exp. group	No. dogs	Protein		DNA (mg/g tissue)
		cytosol (mg/g tissue)	nuclear (mg/g tissue)	
0: castrated	2	13.3 ± 0.5	7.0 ± 2.8	1.32 ± 0.09
1: BPH control	3	45.4 ± 3.5	21.0 ± 1.4	0.20 ± 0.01
2: T-E control	4	34.6 ± 9.5	20.7 ± 3.8	0.35 ± 0.07
3: T-E + OXD	4	33.7 ± 8.6	23.0 ± 4.2	0.41 ± 0.09
4: T-E + OXD + MPA	4	24.2 ± 1.2	21.1 ± 3.4	0.62 ± 0.21

(mean ± SEM)

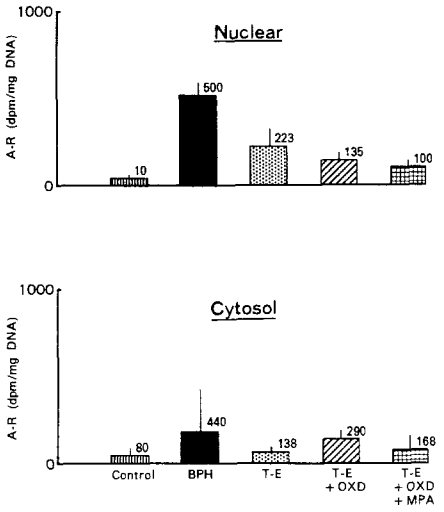

Fig. 4. Nuclear and cytosol androgen receptor (AR) content in the prostate by each experimental group. Expressed by $x10^3$ dpm/mg DNA. A significant decrease in the nuclear AR contents is seen in BPH controls and T-E controls ($P < 0.05$), and between BPH controls and T-E + OXD + MPA-(medroxyprogesterone acetate)-treated groups ($P < 0.01$), but none in other paired comparison and in the cytosol AR

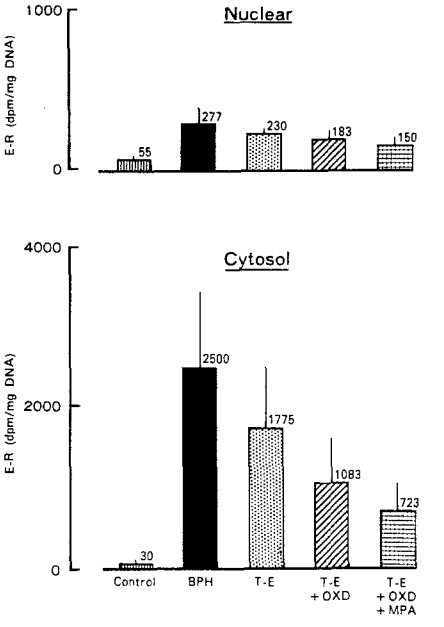

Fig. 5. Nuclear and cytosol estrogen receptor (ER) content in the prostate gland by each experimental group. Expressed by $x10^3$ dpm/mg DNA. The decreasing tendency was observed in both cytosol and nuclear ER contents similarly to those of AR, though a significant difference was not seen. ER contents was highest in BPH controls and lowest in the castrated dogs

Discussion

BPH is a prostatic disease of older age peculiar to men and dogs. Although distinct differences are seen in their histology [7, 15], many researchers have investigated the etiology and the effects of hormonal manipulation in canine BPH. Though most of the recent knowledge on this topic was accumulated from studies on spontanously and experimentally induced canine BPH, few studies have been reported on the effects of anti-androgens, some of which are now commercially available for medical control of the disease.

We investigated the effect of Oxendolone (OXD), alone or in combination with medroxyprogesterone acetate (MPA). OXD is a compound which can be classified as a pure anti-andorgen. As previously stated, this compound exerts its potent anti-androgenic activity mainly by competition with receptor binding of androgens [14, 18], and additionally by its inhibitory effect on the enzyme activity of 5α-reductase, which converts testosterone (T) to 5α-dihydrotestosterone (DHT), without exerting any kind of other hormonal activity such as an androgenic, estrogenic, or gonadotropin increasing action [12, 17].

In this experimental model, we demonstrated the effectiveness of OXD which reduces the size of the gland and causes atrophic glandular elements associated with relatively increased stromal tissue. These effects correlated with a tendency towards a decreased nuclear androgen receptor (AR). However, these changes wer not remarkable compared to the T-E controls.

The reason that we could not demonstrate a significant effect of OXD compared with the T-E control was that the control group itself showed an atrophic tendency in the size and the histological findings (Part I. of this report) and a decline in DHT and AR content in the prostate. It has been elected to use T-E substitution, because deKlerk et al. [4] demonstrated that T had not sufficiently maintained the DHT content in dog BPH which lead to lesser BPH in castrated dogs. At the same time, they also stated that stromal proliferation could be obtained. We attempted to conduct the present experiments in this modified model because we wanted the histology as close as possible to that of human BPH. However, the regression of the prostate was much more excessive than we expected. This situation was the reason for less pronounced differences seen between the treated groups.

The role of estrogen in the pathogenesis of BPH is debated. There has been much experimental and clinical evidence that estrogen stimulation is inevitable in the development of BPH. While Wilson et al. failed to induce BPH in the castrated dog by a long-term administration of DHT [22], BPH could be achieved if the administration were coupled with estradiol (E_2) [3]. Furthermore, the evidence that the canine prostate contains an abundant amount of estrogen receptors [2, 20] and that exogenous estrogen increases in the cytosol androgen receptor [13] as well as the nuclear androgen receptor [20] indicates that estrogen plays an important role in inducing BPH in dogs. Direct proof in humans for the role of estrogens has not been obtained, however a recent study at Johns Hopkins University Laboratory demonstrated that a small number of estrogen receptors were also found in human prostatic tissue [4].

We therefore wished to investigate the synergistic inhibitory effect of an anti-estrogenic compound on BPH

when administered together with an anti-androgen. The anti-estrogen finally chosen was MPA rather than the readily available anti-estrogens, Tamoxifen for instance, which has a slight estrogenic action in dogs which had to be avoided in this experiment. For the reason given in Part I, the medroxyprogesterone acetate (MPA) dosage of 30 mg per dog per week was considered to be anti-estrogenic rather than anti-androgenic in the castrated dog. Acutally, the most pronounced effect was seen in the group treated with OXD combined with MPA. However, it could not be clearly demonstrated whether MPA indeed acted as an anti-estrogenic substance. It may have had an influence as an anti-andorgen, but this could not be known without the availability of another experimental group treated with MPA alone.

Whatever the mechanisms are, it may be of clinical significance that a combination therapy such as applied in these experiments induced a marked shrinkage in an experimentally induced BPH.

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