J. Vet. Med. A **50,** 225–229 (2003)

ISSN 0931–184X

PHYSIOLOGY

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Effect of Testosterone and Oestradiol-17β on Canine Hair Follicle Culture

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With 2 figures and 3 tables

Received for publication: January 22, 2003

Summary

Skin biopsies were taken from four body sites (head, thorax, flank and perineum) of three male entire Beagles and the primary hair follicles were isolated. Culture conditions were established to keep the hair follicles growing for up to 7 days. Additionally, hair follicles were incubated in supplemented medium (containing insulin, transferrin, glutamine and sodium selenite) with or without the addition of testosterone (T) (1, 10 or 100 ng/ml) or oestradiol-17β (E2β) (0.01, 0.1 or 1 ng/ml), respectively and the daily growth of hair follicles was measured. *In vitro* daily growth of hair follicles from the thorax was stimulated by the low concentration of both hormones, but the growth of those from the flank was inhibited by the high concentration of both hormones. Hair follicles from the head were positively influenced by the lowest concentration of T and the medium concentration of E2β. The daily growth of hair follicles from the perineum was not significantly influenced by either hormone.

Introduction

The observation of Hamilton (1942) reveals that sexual steroids play an important role in the regulation of hair growth. The rise of the plasma level of these hormones during puberty mediates the transformation of a fine, short vellus hair into the thicker and longer terminal hair of adults. On the other hand, sexual steroids can be involved in the disruption of hair growth as well. In humans it has been demonstrated, that the local metabolism of testosterone (T) within the hair follicle plays a major role in the pathophysiology of male pattern baldness, also known as androgenetic alopecia (Hoffmann and Happle, 1999; Stenn and Paus, 2001).

Canine endocrine disorders are often characterized by changes of the hair coat quality and the appearance of alopecia (Rhodes, 1990; Rosychuk, 1998). The hair loss starts at predilection areas, as the flank or the perineum, while other body sites like the head or the legs are not affected. Alopecia in the perineum is often associated in the male dog with an oestrogen secreting Sertoli cell tumour and in the flank of the bitch with hormone producing ovarian cysts.

In order to study the effect of steroid hormones on hair growth (without any interference of changing levels in the circulation) it is necessary to separate hair follicles and keep them in organ culture. Important information about the local androgen metabolism has been gained by organ culture of whole hair follicles in several species. However, this method has not been previously reported in the dog.

Therefore the aim of the study was to establish the organ culture of canine primary hair follicles and to test the effect of T and oestradiol-17β (E2β), respectively, on daily growth of hair follicles *in vitro*.

Materials and Methods

Skin biopsies (8 mm diameter) were taken from three male intact Beagle dogs (aged between 9 and 11 months), which were kept at the First Medical Clinic for Horses and Companion Animals, Veterinary University Vienna and for which the permission for animal experimentation had been obtained (GZ 68.205/48-Pr/4/99). The area of the skin from which the biopsies were taken, was shaved 14 days prior to the biopsy, in order to induce the anagen phase of the majority of hair follicles. The skin was cut into small strips (1 × 3 mm) and the epidermis was removed with a scalpel blade. Under a dissecting microscope the composed hair follicles were isolated using watchmaker's forceps and 20G No. 1 injection needles and kept for 1 h in Petri dishes with Eagle balanced salt solution (EBSS) + phosphor balanced salt solution (PBSS) at equal parts and a pH of 7.2.

As soon as possible (within 30 min to 2 h after the biopsy), primary hair follicles were isolated. They were cut proximal to the duct of the sebaceous gland and the distal part was discarded. Each primary hair follicle was placed in one well of a 24-well plate and incubated in 2 ml of supplemented Williams E medium (Life Technologies, Paisley, Scotland, UK; modified according to Hoffmann et al., 1996) at 37°C, 95% air and 5% CO_2.

In pilot experiments the addition of supplements (10 μg/ml insulin, 10 μg/ml transferrin, 2 μmol/ml glutamine, 10 ng/ml sodium selenite; Sigma-Aldrich, Wien, Austria) and of 10% steroid-free, inactivated (56°C, 2 h) canine serum (Promocell

Inc., Heidelberg, Germany) enabled growth of hair follicles *in vitro*. Incubation of hair follicles in non-supplemented medium (without insulin, transferrin, glutamine and sodium selenite) stopped hair growth and led to morphological alterations within 24 h.

The growth of the hair follicles was monitored by daily measurements using a microscope (Labophot 2; NIKON, Kanagawa, Japan) connected to a digital imaging system (Camera CF 15 DSP, software: Image Base Noah, KAPPA, Opto Electronics GmbH., Gleichen, Germany).

Only hair follicles, which were in the anagen phase at the time of preparation, morphologically intact and which had grown more than 0.1 mm within the first 24 h were used for further investigations.

Testosterone (A 6950; Steraloids, Wilton, NH, USA) or E2β (E 9050; Steraloids, Wilton, NH, USA) was added in three different concentrations (1, 10 or 100 ng/ml = 3.47, 34.7 or 347 nmol/l for T and 0.01, 0.1 or 1 ng/ml = 0.037, 0.367 or 3.67 nmol/l for E2β, respectively) to the medium, which was changed every 3 days. Control samples were incubated without hormone addition.

For each dog and each body site, at least three hair follicles were used. To exclude the possibility that the growth of the hair follicles would be caused by a simple progressing of the hair shaft during catagen transformation, six hair follicles were incubated in non-supplemented Williams E medium. These hair follicles did not show any increase in length.

Evaluation of the results

Two identical mixed models (Proc GLM, SAS Release 8.01) were calculated for T and E2β, respectively. Dependent variables were increase in hair follicle growth from days 2 to 7 and independent variables were dog, body site, dose and day.

Results

Altogether 375 hair follicles could be investigated. Hair follicles incubated in supplemented medium showed a marked daily increase in growth (Figs 1 and 2), whereas those incubated in non-supplemented medium displayed hardly any growth (Fig. 2).

The statistical evaluation of the results revealed a significant smaller daily growth of the hair follicles on days 6 and 7, as compared with that of previous days. Comparing the different body sites, it could be seen that without any addition of T or E2β the hair follicles from the flank and the hair follicles from the perineum grew significantly more than those from the head, and those from the flank grew significantly more than those from the thorax. However, there was no significant difference between the growth of hair follicles taken from the head compared with those from the thorax (Fig. 2).

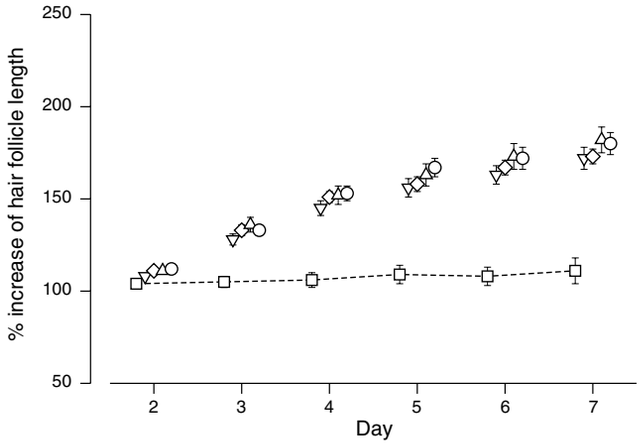

Fig. 2. Percentage (mean ± SEM) of daily growth of canine hair follicles *in vitro*. Biopsies were taken from the head (v), thorax (◇), flank (△) and perineum (○), respectively. The open squares (□) represent the hair growth in samples incubated in non-supplemented medium (without the addition of insulin, transferrin, glutamin and sodium selenite).

On the growth of hair follicles from the head, only the addition of T in its lowest concentration (but not at higher doses) and that of E2β in the medium dose (but not in the others) had a significant positive influence. The daily growth of hair follicles from the thorax was significantly enhanced by the lowest concentration of T, and otherwise significantly reduced by the medium and high concentrations of E2β. On hair follicles from the flank only the high concentration of T had a significantly negative effect on daily growth, the addition of E2β showed this negative effect in both, the medium and the high concentrations. Hair follicles from the perineum were neither influenced by the addition of T nor of E2β in any of the three concentrations.

The arithmetic mean and the standard deviation of the percentages of daily growth of hair follicles (based on individual measurements) are shown in Table 1 for the addition of T and in Table 2 for the addition of E2β. The

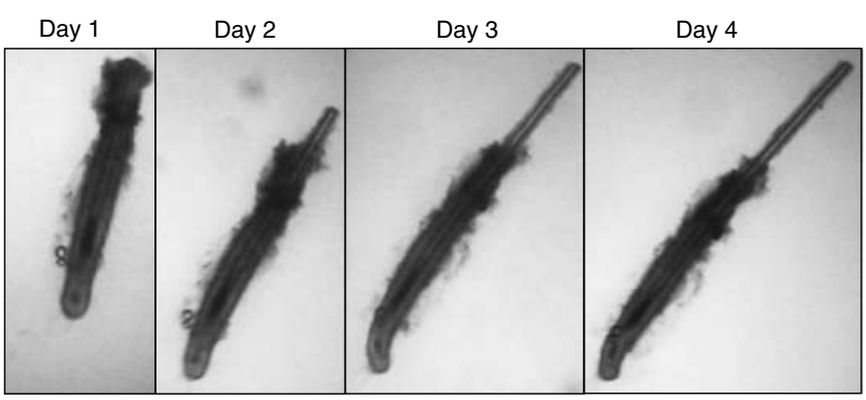

Fig. 1. Microscopic photo (40×) of the daily growth of canine hair follicles *in vitro*.

Table 1. Percentage of daily growth of canine hair follicles *in vitro* (mean ± SD, n = average number of observations. Each incubation contained one hair follicle, whose growth was measured daily.) without or with the addition of testosterone (1, 10 or 100 ng/ml = 3.47, 34.7 or 347 nmol/l) to the medium

		Day of organ culture						
		2	3	4	5	6	7	
Medium	Concentration (nmol/l)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	n
H	O	108 ± 6	131 ± 10	151 ± 15	162 ± 16	170 ± 17	177 ± 19	14
	3.47	109 ± 2	134 ± 7	157 ± 12	170 ± 15	179 ± 18	191 ± 16	9
	34.7	110 ± 5	134 ± 13	156 ± 21	166 ± 25	176 ± 28	187 ± 28	9
	347	112 ± 5	133 ± 11	155 ± 18	166 ± 18	178 ± 21	189 ± 28	14
T	O	112 ± 4	134 ± 12	153 ± 15	161 ± 14	169 ± 17	175 ± 6	18
	3.47	112 ± 5	138 ± 11	156 ± 15	167 ± 19	176 ± 24	184 ± 23	12
	34.7	110 ± 4	131 ± 11	149 ± 13	160 ± 15	168 ± 18	173 ± 19	11
	347	111 ± 4	133 ± 9	151 ± 10	161 ± 11	169 ± 14	175 ± 16	11
F	O	114 ± 4	143 ± 8	161 ± 11	174 ± 16	181 ± 18	189 ± 17	13
	3.47	116 ± 5	139 ± 12	158 ± 11	166 ± 11	174 ± 10	181 ± 9	10
	34.7	113 ± 10	137 ± 17	158 ± 19	169 ± 19	176 ± 18	183 ± 17	9
	347	111 ± 3	131 ± 8	150 ± 10	160 ± 8	169 ± 10	174 ± 9	12
P	O	113 ± 4	136 ± 10	155 ± 15	170 ± 17	179 ± 19	187 ± 19	18
	3.47	109 ± 4	132 ± 14	150 ± 16	162 ± 18	169 ± 17	178 ± 21	13
	34.7	112 ± 6	136 ± 11	155 ± 15	167 ± 17	175 ± 16	185 ± 16	11
	347	114 ± 7	139 ± 12	157 ± 17	172 ± 18	178 ± 25	185 ± 27	12

H, head; T, thorax; F, flank; P, perineum.

Table 2. Percentage of daily growth of canine hair follicles *in vitro* (mean ± SD, n = average number of observations. Each incubation contained one hair follicle, whose growth was measured daily.) without or with the addition of oestradiol-17β (0.01, 0.1 or 1 ng/ml = 0.037, 0.367 or 3.67 nmol/l) to the medium

		Day of organ culture						
		2	3	4	5	6	7	
Medium	Concentration (nmol/l)	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	n
H	O	108 ± 6	131 ± 10	151 ± 15	162 ± 16	170 ± 17	177 ± 19	14
	0.037	112 ± 4	135 ± 9	154 ± 10	162 ± 12	173 ± 13	180 ± 14	12
	0.367	111 ± 3	134 ± 8	154 ± 10	165 ± 10	174 ± 13	182 ± 14	11
	3.670	111 ± 4	135 ± 11	154 ± 12	162 ± 14	170 ± 15	179 ± 17	10
T	O	112 ± 4	134 ± 12	153 ± 15	161 ± 14	169 ± 17	175 ± 16	18
	0.037	112 ± 3	136 ± 3	153 ± 13	165 ± 15	174 ± 16	184 ± 12	11
	0.367	111 ± 5	131 ± 12	149 ± 17	156 ± 18	162 ± 16	168 ± 16	10
	3.670	110 ± 3	128 ± 6	147 ± 11	159 ± 12	166 ± 11	174 ± 12	8
F	O	114 ± 4	143 ± 8	161 ± 11	174 ± 16	181 ± 18	189 ± 17	13
	0.037	113 ± 8	134 ± 11	152 ± 12	163 ± 15	170 ± 14	177 ± 15	10
	0.367	110 ± 5	129 ± 11	147 ± 16	158 ± 17	167 ± 18	175 ± 21	10
	3.670	112 ± 6	135 ± 12	151 ± 14	163 ± 17	174 ± 19	171 ± 21	10
P	O	113 ± 4	136 ± 10	155 ± 15	170 ± 17	179 ± 19	187 ± 19	18
	0.037	111 ± 5	133 ± 7	153 ± 11	162 ± 12	171 ± 17	181 ± 18	13
	0.367	112 ± 4	135 ± 8	157 ± 11	167 ± 14	178 ± 14	183 ± 19	10
	3.670	110 ± 2	133 ± 10	152 ± 13	163 ± 13	171 ± 13	179 ± 13	12

H, head; T, thorax; F, flank; P, perineum.

least square means of the percentages of daily growth of hair follicles within 6 days are given in Table 3 for the addition of both hormones.

Discussion

In the field of hair research, important insights could be gained by studying the growth of hair follicles *in vitro*. Organ culture is regarded as a useful tool for hair research and has been used in humans (Kondo et al., 1990; Hoffmann et al., 1996; Philpott et al., 1996), mouse (Kamiya et al., 1998), sheep (Bates et al., 1997) and horse (Williams et al., 1996). The average daily growth of hair follicles in culture corresponded to that of the respective species *in vivo*.

The present study is the first report of canine hair follicles kept in organ culture. Although the organ culture started with anagen hair follicles, after 7 days all hair follicles were in the catagen phase. A reason for the significantly reduced growth of hair follicles on days 6 and 7 may be that the organ culture conditions were already suboptimal for the full growth *in vitro*.

Table 3. Least square means for the effect of the dose. The figures represent the least square means of the percentages of daily growth of hair follicles within 6 days

	nmol/l	Head	Thorax	Flank	Perineum
T	O	146[a]	150	160[a,b]	155
	3.47	158[b]	155[a]	156[b]	150
	34.7	149	149[b]	156[b]	152
	347	151	150	149[c]	157
E2β	O	146[a]	151	159[a]	155
	0.037	151	153[a]	151	152
	0.367	153[b]	146[b]	146[b]	155
	3.670	151	147[b]	149[b]	148

Different superscript letters indicate significant difference, the significance level was set to $\alpha = 5\%$.
T, Testosterone; E2β, Oestradiol-17β.

Four different body locations of the skin biopsies were selected in order to find differences in hair growth between predilection areas for endocrine-related alopecia (flank, perineum) and other areas, which are rarely or not at all affected (thorax, head).

In the control group, without any addition of T or E2β, the hair follicles from the flank revealed the greatest daily growth *in vitro*. This is in agreement with the *in vivo* results of Gunaratnam and Wilkinson (1983), who found significant greater hair growth in the flank as compared with the head.

In the present *in vitro* study, three different concentrations were chosen for the addition of T or E2β to the culture medium. The medium concentration resembled that of the respective hormone in the peripheral blood of male dogs (Mialot et al., 1988; Peters et al., 2000), the other two concentrations were lower or higher by a factor of 10, respectively.

The statistical evaluation of our results revealed that the daily growth of hair follicles from the perineum was not significantly influenced by the addition of either T or E2β. The lowest concentration of T had a stimulatory effect on hair follicles from the head and the thorax. On the other side, the medium and high concentrations of E2β significantly reduced the growth of hair follicles from the thorax. A marked influence of the addition of T or E2β could be observed in hair follicles taken from the flank. The highest concentration of T and the medium and high concentrations of E2β significantly reduced the daily hair growth *in vitro*.

The negative effect of E2β on hair growth in the dog has been observed already by Eigenmann et al. (1984), who found a bilateral symmetric alopecia in the flank region of an ovariectomized bitch, 2 weeks after she received an E2β implant. The concentration of oestrogen receptors (ER) in the affected skin was six times higher than in the control area. Furthermore, normal hair growth was resumed 6 weeks after removal of the implant. The authors concluded that the alopecia was caused by a local effect of the oestrogen in the skin rather than a systemic hyperoestrogenism.

A similar form of alopecia in the perineum has been found in older male dogs with Sertoli cell tumours, which had elevated levels of E2β in the blood (Peters et al., 2000). However, in our *in vitro* studies with hair follicles from the perineum, the addition of T or E2β had no significant effect on daily hair growth. It could be that the short duration (6 days) of the incubation of hair follicles with the hormone was not sufficient for the development of marked effects.

The hair growth inhibiting effect of E2β is in agreement with the results obtained *in vivo* in mice (Oh and Smart, 1996), where the administration of E2β (50 nmol/ml acetone) onto the back skin lead to the telogenization of the hair follicles.

As expected, hair follicles from the flank (a predilection area of sex hormone-associated alopecia), were affected in their daily hair growth by either hormone. This is in agreement with the investigation on human hair follicles from the frontal scalp, where an inhibitory effect of both, T and E2β, on the daily growth *in vitro* has been observed (Hoffmann et al., 1996).

In another study, using immunohistochemistry, androgen receptor (AR) and ER have been investigated within the epidermis, in the outer root sheath and in dermal papilla cells of the canine hair follicle (Bratka-Robia et al., 2002). The quantitative distribution of receptor-positive cells was compared between seven different body sites (head, thorax, abdomen, flank, back, perineum and shank). The percentage of AR-positive cells in the epidermis was significantly higher in samples from the thorax and the flank. In dermal papilla cells, the percentage of ER-positive cells was significantly lower in samples from the flank. In the outer root sheath, there was no significant difference in the percentage of receptor-positive cells between the locations.

In our investigation using organ culture of hair follicles, there was no clear dose-dependent effect of the sexual steroids on the daily growth of hair follicles *in vitro*. It could be that the shortening of the hair growth cycle under *in vitro* conditions may have obscured the effect of the hormone addition. However, in hair follicles from the flank an inhibitory effect of the administration of T or E2β could be seen.

Furthermore, it is feasible that T itself is not the appropriate effector of growth of canine hair follicles. It could be that other steroid metabolites were necessary, which are formed in the adnexa of primary hair follicles (e.g. secondary hair follicles, sebaceous glands, dermal fibroblasts). These structures were missing in our organ culture of primary hair follicles.

To further elucidate the effect of sexual steroids on the growth of canine hair follicles from the flank *in vitro,* the local steroid metabolism of these steroids in different cells of the hair follicles (dermal papilla cells, dermal fibroblasts) and the expression of steroid receptors and growth factors should be investigated in the future.

Acknowledgements

This work was financially supported by a grant from the 'Jubiläumsfonds der Oesterreichischen Nationalbank' (Project No. 8145).

References

Bates, E. J., P. I. Hynd, N. M. Penn, and M. J. Nancarrow, 1997: Serum-free culture of wool follicles, effects of nutrients, growth factors and hormones. Brit. J. Dermatol. **137,** 498–505.

Bratka-Robia, C. B., M. Egerbacher, M. Helmreich, G. Mitteregger, M. Benesch, and E. Bamberg, 2002: Immunohistochemical localization of androgen and oestrogen receptors in canine hair follicles. Vet. Dermatol. **13,** 113–118.

Eigenmann, J. E., J. Poortmann, and J. P. Koeman, 1984: Estrogen induced flank alopecia in the female dog, evidence for local rather

than systemic hyperoestrogenism. J. Am. Anim. Hosp. Assoc. **20,** 621–624.

Gunaratnam, P., and G. T. Wilkinson, 1983: A study of normal hair growth in the dog. J. Small Anim. Pract. **24,** 445–453.

Hamilton, J. B., 1942: Male hormone is a prerequisite and an incitant in common baldness. Am. J. Anat. **71,** 451–480.

Hoffmann, R., W. Eicheler, A. Huth, E. Wenzel, and R. Happle, 1996: Cytokines and growth factors influence hair growth *in vitro*. Possible implications for the pathogenesis and treatment of alopecia areata. Arch. Dermatol. Res. **288,** 153–156.

Hoffmann, R., and R. Happle, 1999: Die pathogenetische Bedeutung der 5α-Reduktase-Isoenzyme für die androgenetische Alopezie. Hautarzt **50,** 165–173.

Kamiya, T., A. Shirai, S. Kawashima, S. Sato, and T. Tamaoki, 1998: Hair follicle elongation in organ culture of skin from newborn and adult mice. J. Dermatol. Sci. **17,** 54–60.

Kondo, S., Y. Hozumi, and K. Aso, 1990: Organ culture of human scalp hair follicles, effect of testosterone and oestrogen on hair growth. Arch. Dermatol. Res. **282,** 442–445.

Mialot, J. P., M. Thibier, J. E. Toublanc, M. Castanier, and R. Scholler, 1988: Plasma concentration of luteinizing hormone, testosterone, dehydroepiandrosterone, androstenedione between birth and one year in the male dog, longitudinal study and hCG stimulation. Andrologia **20,** 145–154.

Oh, H. S., and R. C. Smart, 1996: An estrogen receptor pathway regulates the telogen-anagen hair follicle transition and influences epidermal cell proliferation. Proc. Nat. Acad. Sci. USA **93,** 12525–12530.

Peters, M. A., F. H. De Jong, K. J. Teerds, D. G. De Rooij, S. J. Dieleman, and F. J. Van Sluijs, 2000: Ageing, testicular tumours and the pituitary-testis axis in dogs. J. Endocr. **166,** 153–161.

Philpott, M. P., D. A. Sanders, and T. Kealey, 1996: Whole hair follicle culture. Dermatol. Clin. **14,** 595–607.

Rhodes, K. H., 1990: Cutaneous manifestations of canine and feline endocrinopathies. Probl. Vet. Med. **2,** 617–626.

Rosychuk, R. A. W., 1998: Cutaneous manifestations of endocrine diseases in dogs. Comp. Cont. Educat. Pract. Vet. **20,** 287–302.

Stenn, K. S., and R. Paus, 2001: Controls of hair follicle cycling. Physiol. Rev. **81,** 449–494.

Williams, D., P. Siock, and K. Stenn, 1996: 13-cis-Retinoic acid affect sheath-shaft interaction of equine hair follicles *in vitro*. J. Invest. Dermatol. **106,** 356–361.