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VEGF and 17-β-estradiol Levels After Tamoxifen Administration in Canine Hepatoid Gland Adenomas and Hepatoid Gland Epitheliomas

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Abstract. *The aim of the present study was to determine the serum levels of vascular endothelial growth factor in animals in the course of pharmacological treatment against perianal gland neoplasms. Research material comprised of tumor tissue samples obtained from 30 dogs and blood drawn from dogs with tumors and control group animals. The neoplasm type was determined in accordance with the relevant WHO classification. Immunoenzimatic determination of VEGF levels in the blood sera was performed. In all studied animals suffering from tumors, pharmacological tamoxifen treatment was administered, at a dosage of 2 mg/kg bodyweight. The medication was administered for one month. In order to monitor the serum levels of 17-β-estradiol and VEGF, blood was drawn from sick animals three times (on the day of the diagnosis, as well as at one and six months after treatment). The VEGF determination assay was performed in accordance with the manufacturer's guidelines for the ELISA. In the studied group, 12 animals were diagnosed with hepatoid gland adenomas and 18 with hepatoid gland epitheliomas. Elevated VEGF levels were observed in the group of dogs with hepatoid gland ephithelioma in comparison with the control group. In the studied groups, a decrease in serum VEGF level and a complete remission of neoplastic lesions was observed one month after administering tamoxifen. The VEGF levels in dogs with hepatoid gland adenoma continued to decline with time. In the case of dogs with hepatoid gland epithelioma, after the initial drop one month after treatment, a rapid increase of the growth factor level was observed, which was significantly higher in animals suffering a relapse of the neoplastic disease (50% of dogs). A significant correlation was observed between 17-β-estradiol and VEGF levels in dogs with hepatoid gland epithelioma on the day of diagnosis (R_{xy}=0.64, p<0.05) and six months after treatment (R_{xy}=0.54, p<0.05). Conclusion: VEGF overexpresion observed six months after tamoxifen treatment may constitute a prognostic factor in terms of the progression of the neoplastic process. The level of VEGF correlates with the level of 17-β-estradiol in serum. Apart from anti-estrogen effects, tamoxifen also demonstrates anti-angiogenic activity.*

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Perianal gland tumors represent approximately 9% of skin tumors diagnosed in dogs. They are most common in older, unneutered male dogs or sterilized female dogs.

Proliferative lesions in this area may originate from three types of glands: anal glands, anal sac glands, and perianal, hepatoid glands (1, 2). They are most often adenomas and adenocarcinomas (3).

Epitheliomas, classified by the World Health Organization as sebaceous and modified sebaceous gland tumors, are also common. They are characterized by low-grade malignancy but can infiltrate adjacent tissues and have a tendency to relapse following pharmacological and surgical treatment (10). Literature confirms that the tumors are hormone-dependent (3, 37). The presence of estrogen receptors (ER) and androgen receptors (AR) was reported both in the perianal and surrounding tissue affected by the neoplastic process (30). Hormone dependence is one of a number of factors contributing to the pathogenesis of perianal tumors. It is believed that another factor participating in oncogenesis may also be a mutation of the gene encoding the p53 protein and growth factor responsible for neoplastic angiogenesis.

Angiogenesis is a process necessary for correct growth and it involves the formation of new blood vessels from the already existing vascular network. Physiological angiogenesis is typically intensified during embryogenesis, ovulation, pregnancy, and in the process of wound healing (6). It also plays a vital part in the development of neoplastic tumors as it conditions their growth. Newly-formed vessels not only provide cancer cells with nutrients and oxygen but are also

responsible for cell migration into the bloodstream, thus increasing the risk of metastasis. Neoangiogenesis is very closely controlled by certain 'positive' and 'negative' regulators. In a mature organism, these are produced in equilibrium, which prevents blood vessel carcinogenesis. When the balance is upset, however, the production or effects of one or many pro-angiogenic factors are intensified and angiogenesis takes place (23). Research results obtained from humans and animals indicate that the main factor stimulating proliferation and migration of vascular endothelial cells is the vascular endothelial growth factor (VEGF). VEGF is secreted by a number of cells: T-lymphocytes, monocytes, macrophages, activated platelets, fibroblasts, and neoplastic cells. VEGF synthesis may also be induced by hormones, other growth factors, and cytokines (15). A separate group of factors related to the synthesis and expression of VEGF includes mutation of tumor-suppressor genes and activation of oncogenes (20).

Tamoxifen has been used in cases of hormone-dependent tumor. It is a non-steroidal, anti-estrogenic agent categorized as one of the selective estrogen receptor modulators. The anticancer effect of tamoxifen is mainly due to its anti-estrogenic activity, but it also facilitates certain alternative therapeutic mechanisms, such as apoptosis induction, inhibiting the proliferation of endothelial cells in the process of angiogenesis through modulation of VEGF synthesis (5, 6).

The aim of the present study was to evaluate VEGF levels in the serum of animals undergoing pharmacological treatment for perianal gland tumors (at the time of diagnosis, as well as after one and six months).

Materials and Methods

The research material comprised tumor samples (biopsy specimens) taken from the anal area of 30 selected male dogs aged between 6 and 14 years, treated at the Department and Clinic of Veterinary Surgery at the University of Life Sciences in Lublin, Poland. The control group comprised 10 healthy dogs, in general good health, aged 2 to 7 years, brought in for sterilization.

Blood was drawn to obtain serum for the determination of 17-β-estradiol and VEGF levels. The level of VEGF in the serum of research and control group animals was determined with use of the ELISA immunoenzymatic assay (Quantikine Canine Immunoassay; R&D Systems, INC Minneapolis, USA, supplier BIOKOM).

All animals with tumors received pharmacological treatment tamoxifen dosed at 2 mg/kg body weight, orally in the form of tablets. The medication was administered for one month.

In order to monitor the serum 17-β-estradiol and VEGF levels, blood was drawn from sick animals three times: on the day of diagnosis, one month after the administration of the hormonal preparation, and six months after the treatment. The level of 17-β-estradiol in the serum of research group animals was determined with use of the ELISA immunoenzymatic assay (Biomerieux, Marcy l'Etoile, France). The VEGF and 17-β-estradiol determination by ELISA assay was performed in accordance with the manufacturer's guidelines.

Biopsy specimens from each tumor were taken for histopathological examinations with the use of a skin trephine of 0.6 cm in diameter. The obtained material was delivered to the Chair of Pathoanatomy at the Department of Veterinary Medicine of the University of Life Sciences in Lublin. The tissue samples were preserved for 24 h in 10% formalin, -stained and histopathologically examined in accordance with WHO histopathological classification of skin tumors (10).

Samples for immunohistochemical tests were placed on silane-coated Super Frost slides (Menzel-Glaser, Braunschweig Germany, DAKO) and kept in an incubator in 56°C for 12 h. Xylene was used to remove paraffin from dried slides which were subsequently placed in solutions of decreasing alcohol concentration and distilled water. Ki-67 antigen expression was assessed using Dako-ARKTM set for the immunohistochemical staining of animal tissues (Animal Research Kit, Dako) reducing the background of non-specific reactions. Mouse monoclonal antibody (clone MIB-1) against Ki-67 protein was used (Dako).

At ×40 magnification, the preparations were examined in terms of Ki-67 antigen expression and an index was calculated corresponding to the percentage of positively stained cells per 500 tumor cells.

A statistical analysis was performed to determine whether significant correlations existed between the VEGF levels in the serum of animals with tumors and the control group, as well as if statistically significant discrepancies were observed in terms of VEGF levels after one and six months from the administration of pharmacological treatment.

The relation between Ki-67 staining for the two types of tumor was calculated with the use of Pearson's χ^2 test. A significant relation was also observed between the 17-β-estradiol levels and VEGF levels in the blood. To verify the normality of the distribution of the obtained results, the Shapiro–Wilk test was used. In comparison of two groups of results, in the absence of a normal distribution whose test function was designated as 'Z', the Mann–Whitney test was used, whereas in those pertaining to more than two groups of results regarding the same type of data measured at different times, the Friedman ANOVA test was applied, with the test function designated as "χ^2 ANOVA".

Results

In the research group, 12 animals were diagnosed with hepatoid gland adenomas and 18 with hepatoid gland epitheliomas, characterized by low malignancy (Tables I and II).

After one month of tamoxifen treatment, tumor remission was observed in all studied dogs. In animals suffering from hepatiod gland adenomas, remission lasted for at least six months after treatment, whereas progression of the disease in the form of individual nodules was observed in over 10 of dogs with hepatoid gland epithelioma.

Expression of the proliferative antigen Ki-67 was observed in most of the studied neoplasms; a significant correlation was reported in terms of the incidence of Ki-67 presence in hepatoid gland adenomas and hepatoid gland epitheliomas. In dogs diagnosed with hepatoid gland epithelioma, the presence of Ki-67 was confirmed in 14 cases (χ^2=5.93, p<0.01).

Table I. *Results of histopathological, biochemical and immunoenzymatic tests in the group of dogs with hepatoid gland adenoma.*

Breed, gender, age in years	Ki-67 staining	Time point 0		Time point 1		Time point 2	
		17-β-estradiol (pg/ml)	VEGF (pg/ml)	17-β-estradiol (pg/ml)	VEGF (pg/ml)	17-β-estradiol (pg/ml)	VEGF (pg/ml)
Dachshund, ♂, 7	+	35	13.72	27	15.71	15	11.03
Mixed, ♂, 13	–	28	14.77	11	12.29	3.5	12.14
German mastiff, ♂, 6	–	12.4	8.61	6.4	5.10	3.1	3.26
Mixed, ♂, 8	–	9.46	1.65	2.21	1.37	1.6	1.02
Dachshund . ♂. 6	+	7.68	14.82	8.60	10.36	6.89	8.61
Mixed, ♂, 8	+	12.40	17.5	9.86	14.8	6.24	10.37
German Shepherd, ♂, 6	–	21	3.36	15.60	2.98	5.02	1.65
Mixed, ♂, 13	–	31	13.27	8.0	12.59	2.79	10.19
Mixed, ♂, 8	–	27	14.78	21.08	12.89	12.08	11.09
German mastiff, ♂, 7	+	8.24	6.85	2.86	6.61	1.3	3.28
Mixed, ♂, 10	–	7.42	19.26	2.02	14.82	1.02	13.92
Mixed, ♂, 9	–	9.26	4.68	2.00	1.24	2.44	0.9

Time point: 0: on the day of diagnosis, 1: one month after treatment administration, 2: six months after treatment conclusion.

Table II. Results of histopathological, biochemical and immunoenzimatic tests in the group of dogs with hepatoid gland epithelioma.

Breed, sex, age in years	Ki 67	Time point 0		Time point 1		Time point 2		Tumor recurrence
		17-β estradiol (pg/ml)	VEGF (pg/ml)	17-β estradiol (pg/ml)	VEGF (pg/ml)	17-β estradiol (pg/ml)	VEGF (pg/ml)	
Mixed breed, ♂, 9	–	41	26.5	18.48	18.68	28.80	28.80	–
Mixed breed, ♂, 13	+	50	23.79	24	11.26	39	32.18	+
Mixed breed, ♂, 8	+	25	18.61	5.78	9.49	7.20	10.81	–
German Shepherd, ♂, 10	+	42	15.71	24.69	6.26	21	59.72	+
Dachshund . ♂. 8	+	28	18.39	23.79	6.67	19.00	36.51	+
Mixed breed, ♂, 13	+	52	27.11	12.00	14.81	28.00	18.39	–
Mixed breed, ♂, 12	+	27	9.37	4.89	5.97	14.6	18.34	–
Dachshund . ♂. 6	+	33	10.37	10	20.19	12	30.11	+
German Shepherd, ♂, 6	+	50	21.09	12	16.52	38	28.38	+
Mixed breed, ♂, 10	+	26	20.19	16.08	13.03	24.79	36.49	+
Mixed breed, ♂, 14	+	35	18.72	27	15.71	33	33.03	+
German Shepherd, ♂, 12	–	24	16.80	16.5	12.02	18.79	18.79	–
Dachshund . ♂. 7	+	50	26.08	24	12.6	32.41	34.8	+
German Shepherd, ♂, 6	+	24	14.20	14.8	10.68	18.00	20.04	–
Terrier, ♂, 10	–	48	12.80	28.8	10.02	32.80	34.89	+
Cocker spaniel, ♂, 9	–	22	10.47	16.80	6.24	18.20	8.24	–
Mixed breed, ♂, 13	+	18	12.40	12.20	4.26	14.20	6.02	–
Mixed breed, ♂, 11	+	36	21.20	14	12.42	33.28	42.80	+

Time point: 0: on the day of diagnosis, 1: one month after treatment administration, 2: six months after treatment conclusion.

The value of VEGF in the control group ranged between 0.1 and 12.53 pg/ml (median=11.14 pg/ml). The median VEGF concentration in the serum of dogs with hepatoid gland adenomas on the day of the first appointment was 13.49 pg/ml (range=1.65-19.26 pg/ml). No statistical differences were observed between VEGF levels in the control group and the growth factor levels in the case of hepatoid gland adenomas. In dogs with hepatoid gland epithelioma, the VEGF level was higher and rayed between 9.37 and 27.11 pg/ml (median=18.50 pg/ml). The VEGF level at diagnosis was significantly higher for dogs with hepatoid gland epithelioma; a breakdown of the obtained results is presented in Figure 1.

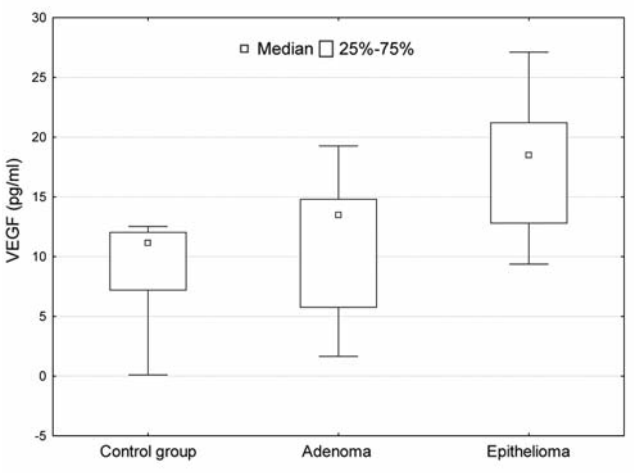

Figure 1. *Vascular endothelial growth factor in serum of dogs with hepatoid gland adenomas and hepatoid gland epitheliomas on the day of diagnosis compared to the control group.*

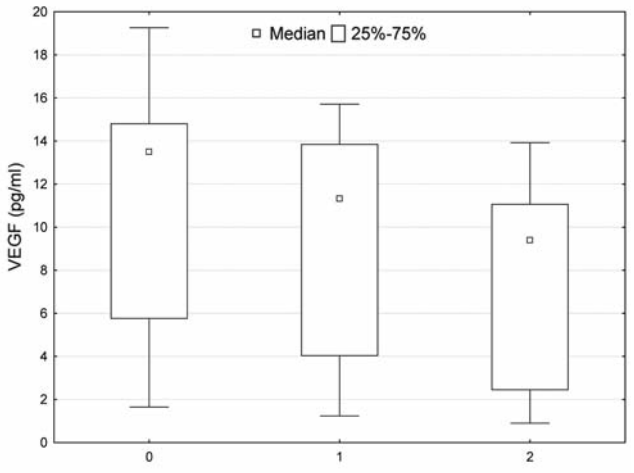

Figure 2. *A breakdown of Vascular endothelial growth factor levels in serum from dogs with hepatoid gland adenomas for each consecutive sampling. Time point 0: at diagnosis; 1: one month after administration of Tamoxifen; 2: six months after administration of tamoxifen.*

By repeatedly monitoring VEGF levels (1 and 6 months after treatment) we observed that the VEGF values for dogs with hepatiod gland adenoma decreased significantly following the pharmacological treatment, from a mean of 13.50 pg/ml to 9.40 pg/ml (χ^2=22.17, p<0.001) (Figure 2).

In hepatoid gland epitheliomas, on the other hand, significant discrepancies in terms of VEGF expression were observed. VEGF values one month after the treatment (mean=11.64 pg/ml) were reduced in comparison to values on the day of the diagnosis (mean=18.50 pg/ml). However, six months after the treatment, the VEGF values were elevated again, with a mean of 29.46 pg/ml, *i.e.* a level higher than that recorded on the day of diagnosis (χ^2=22.17, p<0.001) (Figure 3).

The VEGF levels recorded six months after treatment were also significantly higher in dogs suffering from a relapse of their neoplastic disease (Z=3.47, p<0.001) (Figure 4).

A statistical analysis was performed on the correlation between 17-β-estradiolu and VEGF levels in particular samplings. In the case of hepatoid gland adenoma, no correlation was observed in any period with regard to tamoxifen administration.

A significant relation was observed between the levels of 17-β-estradiol and VEGF in the case of hepatoid gland epithelioma: elevated 17-β-estradiol levels correlated with increased VEGF levels on the day of diagnosis (R_{xy}=0.64, p<0.05) and after six months from tamoxifen administration (Rxy 0.54, p<0.05).

Discussion

Canine perianal glands are modified sweat glands located in the circumanal skin area. In histological terms, they can be described as groups of non-secretory cells similar to hepatocytes. The cells are characterized by sexual dimorphism as in adult females the glands regress to single- gland islets, while in adult males they form gland masses (34).

The presence of AR and ER has been reported superficially on gland cells. These remain under the influence of sex hormones and often it is difficult to distinguish gland hypertrophy from developing adenoma or adenocarcinoma, which are the most common types of tumor occurring in this region. Although the tumors are initially rather small and slow-growing, they can metastasize to regional lymph nodes, organs of the abdominal cavity, or to lungs (2).

In the present study of 30 cases of pathological hypertrophy in the perianal area, 12 cases were diagnosed as hepatoid gland adenoma, while 18 as hepatoid gland ephithelioma. Alhough hepatoid gland ephitheliomas are non-malignant, they can display local invasiveness and a tendency to relapse. The same was confirmed in our research, as six months from the administration of pharmacological treatment, relapse of neoplastic disease was observed in more than half of the dogs.

The factor providing significant information in terms of the tumor's mitotic activity is the extent of cell proliferation (proliferation index by Ki-67). The Ki-67 nuclear antigen is a protein found exclusively in dividing cells during active phases of the cell cycle. Expression of the antigen was studied in a number of canine tumors and its high levels were shown to correlate with neoplastic metastasis and to confer a negative prognosis in cases of canine breast cancer (28). It was reported that Ki-67 is a useful prognostic factor in cases of mast cell tumors (33). Some researchers point-out that in the case of perianal gland neoplasms in dogs, particularly epitheliomas, areas of increased anaplasia can be observed, suggesting transition to high-grade carcinoma. Using proliferation markers (Ki-67) may improve diagnostic

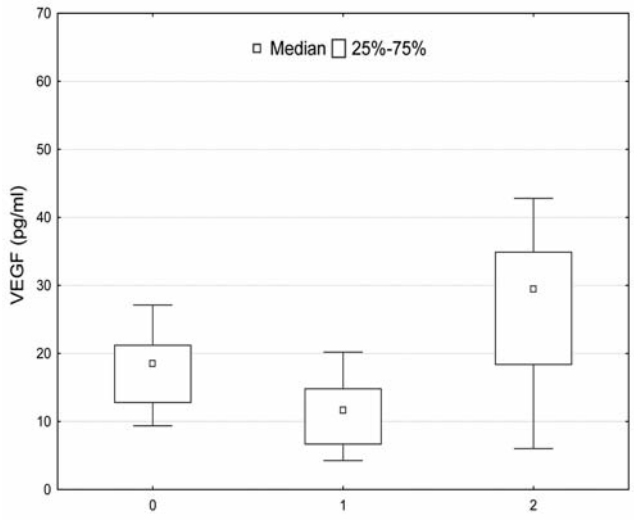

Figure 3. *A breakdown of Vascular endothelial growth factor levels in hepatoid gland epitheliomas for each consecutive sampling. Time point 0: at diagnosis; 1: one month after administration of tamoxifen; 2: six months after administration of tamoxifen.*

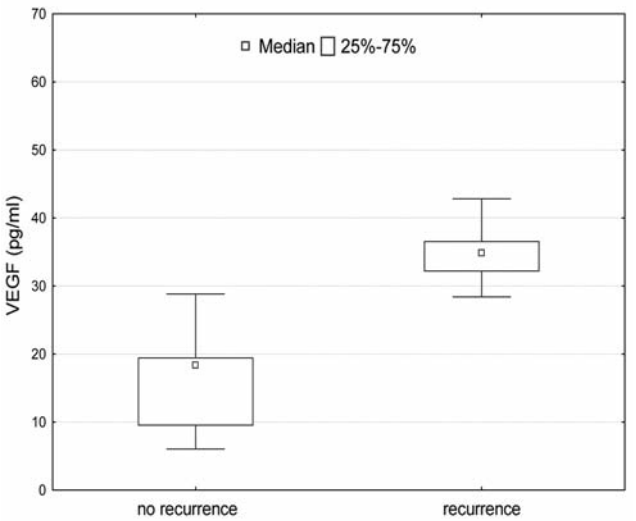

Figure 4. *Breakdown of Vascular endothelial growth factor levels in the course of progressing hepatoid gland epiteliomas.*

criteria in these neoplasms, especially with regard to well-differentiated perianal gland carcinomas (11). Periera *et al*. observed that Ki-67 evaluation is useful in assessing the risk of relapse in canine perianal gland neoplasms (29). The research we conducted indicated a significant correlation in terms of Ki-67 presence in hepatoid gland adenomas and hepatoid gland epitheliomas (χ^2=5.93, p<0.01). In dogs diagnosed with hepatoid gland epithelioma, the presence of Ki-67 was confirmed in 14 cases, possibly indicating the tendency for transformation into a malignant tumor, a fondling similar to that reported by Pereira *et al*.

Perianal gland neoplasms mainly affect unneutered dogs over six years old and the presence of AR in tumor tissue indicates hormone dependence of the neoplasms (26, 30, 37). Apart from androgens, estrogens also play an important part in the process of oncogenesis. Literature suggests that estrogens, through reactions with receptors located in gland cell nuclei, act as promoters of neoplastic cells. Available literature pays considerable attention to the assessment of estrogen levels and their impact on the elevation of VEGF levels, which is in turn responsible for angiogenesis. Most published studies on this topic pertain to breast cancer, both in women and female dogs. Estrogens increase VEGF expression in hormone-dependent tumors in women and consequently lead to the formation of microvasculature necessary for neoplastic expansion and metastasis (9, 13, 19).

The relation between angiogenesis, VEGF, estrogens and their receptors has been confirmed in studies on *in vitro* cell cultures. Elevated expression of VEGF and VEGFR2 receptor is regulated by estrogens (14, 19, 24, 27). Increased VEGF expression in cases of breast cancer in women was related to deteriorating clinical results and the response to hormone therapy (8). A relation was also reported between VEGF levels in peripheral blood and the severity and extent of breast cancer metastasis, which confirms the participation of the growth factor in stimulating tumor growth (18). VEGF was also singled-out as an independent prognostic factor in terms of the risk of relapse and survivability of patients with breast cancer (7).

VEGF expression was also reported in simple mammary gland adenocarcinomas in dogs, which indicates that VEGF has an autocrine function stimulating tumor cell growth (1). It was also reported that VEGF can serve as a marker of malignancy in breast cancer in dogs (35).

Articles have also been published where no correlation between VEGF expression and pathological features was observed in breast tumors in dogs. It was reported that VEGF is independent of the histological type of neoplasm and tissue invasion or local metastatic capacity (32).

Our study revealed, elevated VEGF levels, compared to the control group, in the group of dogs suffering from hepatoid gland epithelioma and the result was statistically significant. Research indicates that VEGF overexpression (above 18.5 pg/ml) in the serum of dogs on the day of diagnosis may indicate a possibly malignant process, but may not be treated as a prognostic factor in hepatoid gland adenoma.

Due to the hormone dependence of breast tumors in women, auxiliary tamoxifen therapy is typically administered. The preparation is confirmed to actively inhibit angiogenesis. In the 1990s, it was included by Folkman in

the so-called "Navy Protocol" treatment he administered to a dog suffering from cancer (16).

Studies were conducted into the impact of administering steroid hormones and antiestrogens on the level of VEGF in breast tumor cells in women (8). Administration of tamoxifen was reported to significantly inhibit VEGF induction and cause anoxia in tumor cells, which resulted in reduced angiogenesis (21, 31). Similar results were obtained by Heer *et al*. They concluded that estrogens may stimulate VEGF expression and that performing mastectomy in combination with anti-estrogen treatment may reduce angiogenic potential, therefore improving the prognosis of patients with estrogen-dependent tumors (12). This effect was observed in animal breast cancer models *in vivo* and correlated with a reduced pace of tumor growth.

Despite the widespread use of tamoxifen in the treatment of breast tumors in women, its application in cases of breast cancer in dogs has been proved ineffective or caused complications such as hypertrophy of the vaginal mucosa and pyometra (17, 36).

Attempts have also been made to treat perianal gland tumors. In 1998, it was reported that administration of tamoxifen caused remission of this type of tumor (17). However, there are no data in terms of the mechanisms of the medicine's activity. Therefore, an attempt was made to administer the treatment to the studied groups of animals while simultaneously studying processes which influence tumor regression.

In all studied groups, a statistically significant decrease in terms of VEGF levels in the serum and complete remission of neoplastic lesions was observed one month after the administration of tamoxifen. In the case of hepatoid gland adenoma, as time passed from the end of the treatment, serum VEGF levels continued to become significantly lower. Meanwhile, in hepatoid gland epithelioma, after the initial VEGF decrease one month after the treatment, the levels of the growth factor increased rapidly in all dogs and the increase was significantly higher in animals undergoing a relapse of the disease. It can, therefore, be hypothesized that overexpression of VEGF observed six months after tamoxifen treatment may constitute a prognostic factor in terms of the progression of the neoplastic disease.

In dogs undergoing treatment, the relationship between VEGF and the level of 17-β-estradiol in blood serum was also studied. It was demonstrated that such a correlation occured only in the case of hepatoid gland epitheliomas. A decrease in 17-β-estradiol levels one month after treatment caused a drop in serum VEGF levels, whereas an increase in 17-β-estradiol levels six months after treatment was accompanied by an increase in VEGF levels. These results allow us to speculate that both 17-β-estradiol and VEGF levels may prove to be a viable prognostic factor. Moreover, tamoxifen, apart from antiestrogen activity, may also have antiangiogenic effects. It is a good preparation for non-invasive treatment of benign perianal neoplasms in dogs, whereas successful therapy in cases of hepatoid gland epithelioma is likely to require prolonged administration of tamoxifen or repeat treatment.

Measurements of VEGF concentrations may facilitate the assessment of the efficacy of antineoplastic therapy. Manders *et al*. demonstrated that elevated VEGF in tumor tissue cytosol prior to treatment corresponded to less favorable results of hormone treatment (22).

It has been observed that certain neoplastic cells are immune to antiestrogen and antiprogestagen treatment. It was also reported that such substances may in fact stimulate tumor growth (4). It was suggested that in the case of such neoplasms, further proliferation is influenced by VEGF (22, 25). Furthermore, clinical studies in humans indicated that neoplasms characterized by low VEGF levels and absence of the ER do not react to hormonal treatment or tend towards early relapse of the disease (7). Therefore, tamoxifen treatment as a monotherapy, particularly in the case of malignant tumors, may prove ineffective and should be combined with antiangiogenic treatment (7).

Currently, the treatment of patients suffering from hormone-dependent tumors involves the use of monoclonal antibodies against VEGF (bevacizumab) in combination with hormonal drugs. The administration of combined antiestrogen and antiangiogenic treatment inhibits tumor growth and may constitute a novel therapeutic approach to the regression of hormone-dependent tumors in humans (27).

It seems viable to suggest also combining hormonal and antiangiogenic therapy in the treatment of neoplasms in dogs, although this would require further clinical studies.

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