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ORIGINAL PAPER

EVALUATION OF SERUM AND TISSUE MAGNESIUM, VASCULAR ENDOTHELIAL GROWTH FACTOR, AND OSTEOPONTIN LEVELS IN DOGS WITH MAMMARY TUMORS WITH/WITHOUT PULMONARY METASTASES AND IN HEALTHY DOGS*

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Abstract

Mammary tumors in female dogs are usually malignant and tend to metastasize to distant organs, especially to regional lymph nodes and lungs. Radiography is the standard diagnostic method to detect pulmonary metastases in these animals. Magnesium (Mg), vascular endothelial growth factor (VEGF), and osteopontin (OPN) levels have been used in recent studies to make prognoses of human breast cancer. To the best of our knowledge, however, there are not many studies that have been performed on this subject, and there is no study on animals in which the three indicators are scrutinized together. The aim of this present study is to evaluate Mg, VEGF, and OPN levels in healthy dogs and in dogs with mammary tumors with/without pulmonary metastases, and to investigate the alterations of these parameters in the serum and tissue samples of dogs with mammary tumors in connection with the histological tumor type and tumor grade. Mammary tumor groups were designed according to the presence of pulmonary metastasis in radiography; group M1 consists of 20 dogs with metastatic mammary tumors to the lung, and group M0 consists of 20 dogs with nonmetastatic mammary tumors. Ten clinically healthy dogs composed group H. The dogs represented different breeds and ages. Three-view thoracic radiographs were taken to determine any metastasis in lungs by digital radiogra-

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phy. Magnesium, VEGF, and OPN were determined in the mammary gland samples and blood serum of 40 dogs with malignant mammary tumors and in 10 healthy dogs. The magnesium levels were measured by atomic absorption spectrophotometry, both in the tissue and serum samples. Also, the tissue and serum VEGF and OPN levels were determined by ELISA with commercially available kits. The tissue Mg levels in the M0 group were significantly (P<0.05) higher than in group H. However, the serum VEGF level was significantly associated with a tumor type. Additionally, the serum OPN levels exhibited a tendency to be higher in dogs with mammary tumors with pulmonary metastases, grade 3, and carcinosarcoma. It is concluded that Mg, VEGF, and OPN could have practical use for diagnosing and understanding the pathophysiology of CMT.

Keywords: canine mammary tumor, magnesium, osteopontin, vascular endothelial growth factor.

INTRODUCTION

Mammary tumors are the most common neoplasms in intact female dogs. Carcinomas generate 95% of the malignant mammary tumors (Misdorp 2002). Metastases of malignant mammary tumors occur in the lymph nodes, lungs, kidney, and liver (Baba, Catoi 2007). Radiography is still performed to diagnose pulmonary metastases, and lungs are the most common site for distant metastasis of canine mammary tumors – CMT (Glasspool, Evans 2000). Many factors, such as a breed, progestin treatment, ovariohysterectomy status, genetic mutations, obesity, and diet have been claimed to increase CMT risk (Sorenmo 2003).

Magnesium (Mg) is a mineral that participates in cell proliferation, inflammation, energy metabolism, nucleic acid metabolism, protein synthesis, DNA replication, cytoskeletal activation, and antioxidant metabolism (Castiglioni, Maier 2011, Blaszczyk, Duda-Dhodak 2013, Mendes et al. 2017). Magnesium in the body is mainly deposited in the skeletal system, muscles, and bones, while the rest of Mg accumulates in intracellular structures and extracellular fluid compartments (Aikawa 1978). Approximately 20-30% of serum Mg is bound to proteins, while a large amount of Mg is ionized and biologically active (Studzinski et al. 2006). Magnesium is also effective in the progression of human breast cancer (Mendes et al. 2017). The process of breast carcinogenesis is dependent on the accumulation of Mg from blood and nonneoplastic tissues to neoplastic cells (Abdelgawad et al. 2015). It has been reported that pulmonary metastases more often develop in Mg-deficient mice in comparison with the controls due to the intense inflammatory response in the presence of Mg deficiency (Nasulewicz et al. 2004).

Carcinogenesis and neoplastic progression are related to the inflammatory alterations in the tumor microenviroment (Carvalho et al. 2016). Vascular endothelial growth factor (VEGF) is an important cytokine in angiogenesis and tumor progression (Chakraborty et al. 2008). Osteopontin and VEGF can function in synergy, and high levels of both proteins correlate with angiogenesis, clinico-pathological parameters, and a poor prognosis (Ramchandani,

Weber 2015).

Osteopontin (OPN) is a multifunctional cytokine, weighting 41 to 75 kDa. It has a major role in various processes, such as bone remodelling, immune--regulation, inflammation, vascularization and carcinogenesis (Hao et al. 2017). The presence of OPN around the tumor is related to tumor growth, metastasis, and consequently, to a poor prognosis (Shevde, Samant 2014). Osteopontin is expressed in several tissues in humans, but over-expression is usually seen in multiple cancer types, especially in lung cancer, with a strong potential of metastasis and invasion (Shi, Wang 2017). If the OPN expression is high in the primary tumor in human breast cancer or other cancer types, the presence of early metastasis and a poor prognosis are likely expected (El-Tanani et al. 2006).

The aim of the present study was to evaluate the Mg, VEGF, and OPN levels in healthy dogs and dogs with mammary tumors with/without pulmonary metastasis and to investigate the alterations of these parameters in serum and tissue samples.

MATERIALS AND METHODS

The animal care protocol and experimental procedures in this study were approved by the Local Ethical Committee of Animal Experiments (Approval Number: 2018/01).

Ten healthy (H) dogs and 40 dogs with malignant mammary tumors, of different breeds, were enrolled in the study. Mammary tumor groups were designed according to the presence of lung metastasis in radiography; group M1 consists of 20 dogs with lung metastatic mammary tumors, group M0 consists of 20 dogs without lung metastatic mammary tumors (Figure 1). Three-view thoracic radiographs were taken to determine the metastasis on lungs by digital radiography (DR, Ekovia, Korea) – Figure 2. Pixel-flux Software program was used to calculate the size of the lung metastasis. The mammary tumors were histopathologically examined and divided into two groups as carcinoma and carcinosarcoma. The dogs that had multiple

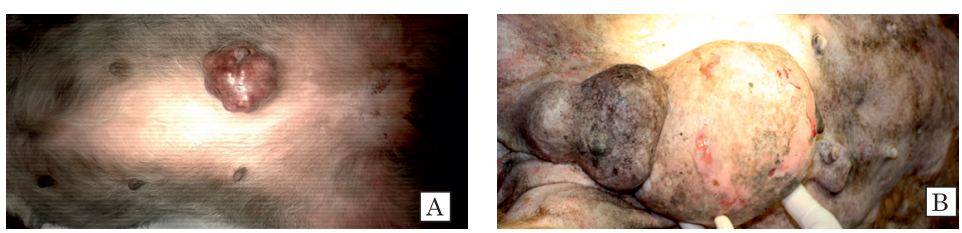

Fig. 1. Dogs with mammary tumor: A – Pekingese, mammary tumor less than 3 cm, no pulmonary metastasis, B – Cocker Spaniel, mammary tumor greater than 10 cm, had pulmonary metastasis

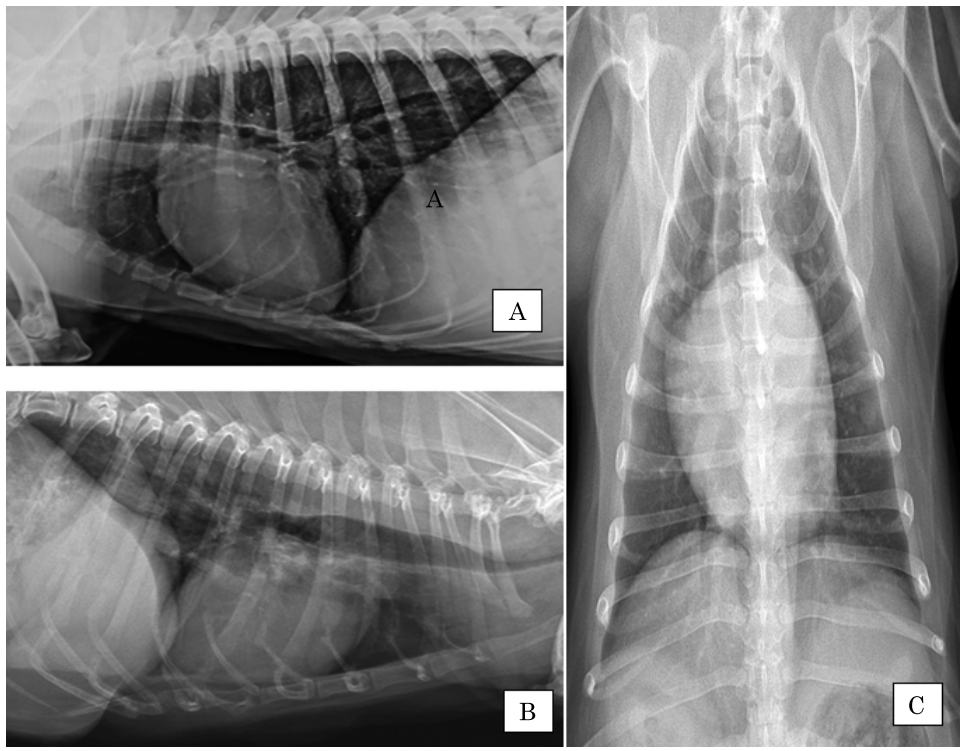

Fig. 2. Three-view thoracic radiography images of dogs with mammary tumors with pulmonary metastasis: A – right laterolateral position, B – left laterolateral position, C – ventrodorsal position

histological tumor types were not included in the study. The histological grading of the tumors consisted of Grade 1, Grade 2, and Grade 3, according to the researchers' report (GOLDSCHMIDT et al. 2011).

Blood samples were collected into clot separator tubes, and they were centrifuged at 4°C with a 3500 cycle for 15 min in order to obtain the serum samples. Mammary gland samples were supplied from healthy dogs during prophylactic mastectomy. Tumor samples were obtained from the center of the mass, immediately after complete mastectomy. All tissue samples were collected into two separate Eppendorf tubes.

Serum and tissue magnesium levels were determined by the method of atomic absorption spectrophotometry (MARCZENKO, BALCERZAK 2000). A NexION® 1000 ICP (PerkinElmer, USA) mass spectrometer was used for the Mg analysis. Other tissue samples were handled with PBS (PH 7.4) (1 ml PBS for 100 mg tissue). They were homogenized in a grinder and then centrifuged for 20 min at 2000-3000 r.p.m. At the end of this procedure, supernatant was collected into a sterile Eppendorf tube. Serum and homogenized tissue samples were stored at -20°C until enzyme-linked immunosor-

bent assay (ELISA) were applied. The OPN and VEGF levels in the tissue and blood serum were determined with commercially available kits according, to the manufacturers' protocols (OPN Catalogue no: 201-15-0618, VEGF Catalogue no: 201-15-2415, Sunred Biological Technology, Shangai, China).

Magnesium, VEGF, and OPN were determined in the mammary gland samples and the blood serum of 40 dogs with malignant mammary tumors and 10 healthy dogs. Also, the serum and tissue Mg, VEGF, and OPN levels were evaluated in terms of the histological type and grading.

Statistical analyses

SPSS 13.0 software was used for the statistical analyses. One-way ANOVA and the Duncan tests were used to evaluate the significance between the groups (M1, M0, H) in terms of the tissue and serum Mg, VEGF, and OPN levels. Also, a t-test was performed to evaluate the tissue and serum Mg, VEGF, and OPN parameters related to the tumor types. Additionally, one-way ANOVA and Duncan tests were used to evaluate the significance of the tissue and serum Mg, VEGF, and OPN related to histological grading. The significance level was accepted as $P<0.05$.

RESULTS AND DISCUSSION

The mean ages of the dogs in groups M1, M0, and H were detected as 11.50±0.73, 10.65±0.46, and 2.90±0.50 years, respectively. The mean values for the size and number of lung metastasis in the M1 group were 5.89±0.38 mm and 5.57±0.57 nodules, respectively.

The mean values of tissue and serum Mg, OPN, and VEGF related

Table 1

Mean values of tissue and serum Mg, OPN, and VEGF, and their significance related to groups

Specification		M1 (n=20)	M0 (n=20)	H (n=10)	Significance
		mean±SE	mean±SE	mean±SE	
Tissue	Mg (μg g^{-1})	$119.01±9.33^{ab}$	$159.91±23.40^{b}$	$72.04±20.10^{a}$	**
	OPN (ng ml^{-1})	4.64±0.46	4.33±0.38	4.05±0.71	ns
	VEGF (ng L^{-1})	36.22±1.56	37.05±0.98	36.89±0.88	ns
Serum	Mg (μg g^{-1})	20.74±0.65	20.41±0.69	19.78±0.60	ns
	OPN (ng ml^{-1})	$2.10±0.50^{b}$	$0.55±0.18^{a}$	$1.66±0.90^{ab}$	*
	VEGF (ng L^{-1})	17.088±1.37	16.55±1.23	13.87±1.50	ns

Data were statistically analyzed using one-way ANOVA and Duncan tests. Data that showed significant differences in one-way ANOVA test: * P=0.05, ** P<0.05, ns – not significant, M0 – dogs with mammary tumors without lung metastasis, M1 – dogs with mammary tumors with lung metastasis, H – healthy dogs, SE – standard error

to groups are presented in Table 1. The mean value of the tissue Mg level in the M0 group (159.91±23.40 μg g^{-1}) was significantly higher than group H (72.04±20.10 μg) – $P<0.05$. The mean values of the serum OPN level were not significant between group H and dogs with mammary tumors ($P<0.05$). But the mean serum OPN level has a tendency ($P=0.05$) to be higher in the M1 group (2.10±0.50 ng ml^{-1}) than in the M0 group (0.55±0.18 ng ml^{-1}).

Dogs with mammary tumors were histologically classified into two main groups; the number of dogs with carcinoma and carcinosarcoma were 30 and 10 bitches, respectively. Also, the number of dogs affected by the subtypes of carcinoma were as follows: simple carcinoma ($n=14$), complex carcinoma ($n=7$), solid carcinoma ($n=3$), ductal carcinoma ($n=2$), and in situ carcinoma ($n=1$). All carcinoma types were evaluated together in the carcinoma group. According to the main classification, the mean values of tissue and serum

Table 2

Mean values of tissue and serum Mg, OPN, and VEGF and their significance related to tumor types

Specification		Carcinoma ($n=30$)	Carcinosarcoma ($n=10$)	Significance
		mean±SE	mean±SE	
Tissue	Mg (μg g^{-1})	139.09±11.11	140.57±40.91	ns
	OPN (ng ml^{-1})	4.36±0.35	4.85±0.59	ns
	VEGF (ng L^{-1})	36.72±1.13	36.38±1.39	ns
Serum	Mg (μg g^{-1})	20.86±0.57	19.71±0.71	ns
	OPN (ng ml^{-1})	0.73±0.14^a	1.68±0.47^b	*
	VEGF (ng L^{-1})	17.81±1.05^a	13.84±1.54^b	**

Data were statistically analyzed using a *t*-test. Data that showed significant differences in *t*-test: * $P=0.05$, ** $P<0.05$, ns – not significant, SE – standard error

Mg, OPN, and VEGF are presented in Table 2. The mean level of serum VEGF in the carcinoma group was higher than the carcinosarcoma group ($P<0.05$). The mean serum OPN level exhibited a strong tendency to be higher in the carcinosarcoma group versus the carcinoma group ($P=0.05$). The mean values of serum Mg and tissue VEGF, OPN, and Mg were not significant related to the tumor types ($P>0.05$).

The number of the dogs that represented grade 1, grade 2, and grade 3 of cancer were 7, 17 and 16, respectively. The serum and tissue Mg, VEGF, and OPN levels were not significantly associated ($P>0.05$) with histological grading (Table 3). However, there was a tendency ($P=0.06$) for the mean serum OPN levels to be higher in grade 3 than grade 1 (Table 3). Although the number of bitches in the study was low, enough data were obtained to be processed statistically.

The results obtained in this study revealed changes in tissue Mg between the dogs suffering from mammary tumors and healthy dogs. In line with

Table 3

Mean levels of tissue and serum Mg, OPN, and VEGF related to histological grading

Specification		Grade 1 (n=7)	Grade 2 (n=17)	Grade 3 (n=16)	Significance
		mean±SE	mean±SE	mean±SE	
Tissue	Mg (μg g^{-1})	150.54±15.6	119.97±11.44	155.31±28.92	ns
	OPN (ng ml^{-1})	4.97±1.09	4.50±0.50	4.39±0.44	ns
	VEGF (ng L^{-1})	40.26±1.61	36.47±1.07	35.23±1.76	ns
Serum	Mg (μg g^{-1})	20.91±0.80	20.95±0.85	20.03±0.67	ns
	OPN (ng ml^{-1})	0.34±0.11^{a}	0.85±0.22^{ab}	2.15±0.64^{b}	*
	VEGF (ng L^{-1})	15.02±1.86	17.10±1.57	17.30±1.36	ns

Data were statistically analyzed using one-way ANOVA and Duncan tests. Data that showed significant differences in one-way ANOVA test: * P=0.06, ** P<0.05, ns – not significant, SE – standard error

other research (Brodzki et al. 2004*a*, *b*), the Mg level in the neoplastic tissue without metastasis was higher than in the mammary tissue of healthy dogs in the present study. However, the Mg concentrations did not reveal a significant difference between the groups of CMT according to the presence of pulmonary metastases. It was hypothesized that these data were a result of the affinity of Mg to the neoplastic tissue. Similar to Brodzki et al. (2004*b*), the mean level of serum Mg was not significantly associated between group H and dogs with mammary tumors. An accumulation of Mg in the neoplastic tissue caused increased levels of serum Mg, as Abdelgawad et al. (2015) reported. In addition, tissue and serum Mg levels were not significant in terms of tumor types because accumulation of Mg to neoplastic tissue was not depend on tumor types.

In CMT, the serum VEGF levels are positively correlated with a poor prognosis and worse clinical stage. It usually increases in malignant infiltrative tumors and it is considered a marker of tumor progression and metastasis (Queiroga et al. 2011). Kato et al. (2006) reported that the serum VEGF level in dogs with mammary tumors was significantly higher than those of healthy dogs. Also, Raposo-Ferreira et al. (2016) stated that there were significant differences in the comparison of nonmetastatic and metastatic mammary carcinomas with normal mammary tissues (P<0.0287 and P<0.0040, respectively). The results obtained in this study exhibited that serum and tissue VEGF levels were not significantly associated with the presence of the tumor or pulmonary metastases. It was hypothesized that controversial results have been achieved because of the presence of noninfiltrating mammary tumors in CMT groups.

Osteopontin plays a key role in lymphatic metastasis in human breast cancer (Allan et al. 2006). Also, OPN is associated with apoptosis, invasion, and metastasis in lung adenocarcinoma (Stemberger et al. 2014). Additionally, OPN has been regarded as a prognostic and diagnostic marker for several

cancer types (Shevde, Samant 2014). There was a tendency (P=0.05) in the mean serum OPN levels of the M1 group to be higher than in group M0. In accordance with the previous reports (Allan et al. 2006, Stemberger et al. 2014), the mean levels of OPN in the M1 group increased because of the important role of OPN in mammary tumor progression and affinity to lung cells.

In accordance with Abdelgawad et al. (2015), who studied breast cancer, significant differences were not observed in the tissue and serum Mg levels related to histological grading. Similar results were obtained due to the affinity of Mg for neoplastic tissue, regardless of histological grading of the mammary tumor.

In line with Nieto et al. (2007), who studied intratumoral VEGF expression in humans with breast cancer, the serum and tissue VEGF levels were not significantly associated with the histological grade and character of the tumor (metastatic or nonmetastatic) in the present report. It is thought that similar results can be obtained due to the similarity of CMTs and breast cancer in humans.

Weber et al. (2011) reviewed the clinical role of OPN and concluded that it was related to the tumor stage, tumor grade, and early tumor progression. However, Pang et al. (2003) reported that the expression of osteopontin-c correlated with lymph node metastasis, and advanced the TNM stage and histologic grade in breast cancer patients. In contrast with the previous reports (Pang et al. 2003, Weber et al. 2011), the tissue OPN levels were not associated with histological grading in the present study. However, grade 3 the serum OPN levels tended to be higher than grade 1 in terms (P=0.06). Similar to Weber (2011), who reported that OPN was associated with a higher tumor stage or grade, the highest mean serum OPN level was measured in grade 3. Thus, a clinical follow-up should be performed considering the survival time in dogs with grade 3 mammary tumors.

The serum and tissue Mg levels revealed insignificant results (P>0.05) related to tumor types (carcinoma and carcinosarcoma) in the present study. Nasulewicz et al. (2004) investigated Mg deficiency in lung carcinoma, mouse mammary adenocarcinoma, and colon adenocarcinoma cells in vivo and in vitro. They stated that a low Mg status affects tumor growth. Additionally, they reported that a low magnesium status had a deleterious effect on tumor metastasis (Nasulewicz et al. 2004). However, to the best of our knowledge, no information was achieved about the Mg concentration in subtypes of mammary tumor.

Das et al. (2013) reported that OPN had a functional role, to increase the expression of mesenchymal markers in humans. Also, Saad et al. (2017) investigated the immunohistochemical expression of OPN in canine mixed mammary tumors (CMMTs). The mean serum OPN level tended (P=0.05) to be higher in the carcinosarcoma group, as the researchers (Das et al. 2013, Saad et al. 2017) noted, because OPN was localized in the mesenchymal elements of CMMTs.

Moschetta et al. (2015) evaluated the serum concentration of VEGF by ELISA in CMT, and reported that VEGF was associated with a worse clinical stage, a poor diagnosis/prognosis, and a lower survival rate. It was usually increased in cases of more malignant tumors with infiltrative growth. However, Queiroga et al. (2011) suggested that histological tumor types (in situ carcinoma, complex carcinoma, solid carcinoma, tubulopapillary carcinoma, carcinosarcoma) were not associated with VEGF expressions in dogs with malignant tumors. In contrast to Queiroga et al. (2011), the serum VEGF levels were significantly associated with histological tumor types in the present study ($P<0.05$). Also, the serum VEGF levels in carcinoma were higher than in carcinosarcoma. It has been suggested that the contrary results were obtained due to the presence of different numbers of affected dogs and the general classification of histological tumor types in the present study.

In conclusion, due to the affinity of Mg in tumor-growing tissue, increased tissue Mg concentrations were determined in CMT without pulmonary metastasis. However, the serum OPN could have practical application in the determination of a deteriorating clinical profile with regard to histological tumor types, tumor stage, and the presence of metastases. In addition, it has been demonstrated that the serum VEGF is capable of differentiating carcinoma and carcinosarcoma cases. It is hypothesized that Mg, VEGF, and OPN would have a practical use in the diagnosis and understanding of the pathophysiology of CMT.

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