Non-Invasive Assessment of Sentinel Lymph Nodes That Drain the Tumoral Mammary Glands in Female Dog

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Abstract

Mammary gland tumours occupy a significant place in the pathology of this species. Female dogs that are not spayed after their first heat cycle have a higher predisposition of developing mammary tumours. Most tumours metastasize at distance via the lymphatic system. In these conditions, the sentinel lymph nodes of the mammary glands must be assessed prior to surgical treatment.

Considering the insufficient usage of non-invasive investigative methods of the sentinel lymph nodes, the aim of this study is to describe the sonographic anatomy of the lymph nodes that drain the mammary gland tumours in female dog.

Twelve dog females presenting tumours of the cranial and caudal abdominal mammary glands (A1 and A2), inguinal mammary gland (I) and cranial thoracic mammary gland (T1) were examined (group I). In addition, a control group composed of eight dog females was used (group II). The axillary and superficial inguinal lymph nodes were evaluated using an algorithm composed of gray-scale ultrasound, Doppler technique, contrast enhanced ultrasound (CEUS) and real time elastography. Surgical excision of the sentinel lymph nodes was performed and samples for histopathological examination were taken.

The following ultrasonographic findings revealed on gray-scale examination were suspected for the metastatic infiltration: hypoechoic pattern, round shape, hilum absence and heterogeneity. Doppler technique showed an aberrant and mixed vascularisation of the lymph nodes, while the CEUS revealed incomplete enhancement of lymph nodes parenchyma. On real time elastography, the presence of blue areas in more than 50% from the lymph nodes parenchyma led us to conclude that the lymph node stiffness was caused by metastatic infiltration. Histopathological examination confirmed the presence of the metastatic infiltration in 97% of the examined lymph nodes.

The algorithm composed of gray-scale ultrasound, Doppler technique, CEUS and real time elastography proved to be efficient in diagnosing the metastatic infiltration of sentinel lymph nodes of mammary gland. Further studies are needed to validate the proposed algorithm.

Keywords *ultrasonography, lymph nodes, female dog, mammary gland tumour*

INTRODUCTION

Neoplasia is a global reality, which is rapidly growing in many countries, both in human and veterinary medicine. In female dog, there is an increasing incidence of mammary gland

tumour, one of four unsprayed female developing malignant disease (Cassali *et al.*, 2014; Stan *et al.*, 2010). It is also important to note that a high number of practitioners, have reported in recent years the diagnosis of malignant melanomas and

lymphomas in veterinary medicine (Goldberg *et al.*, 2004, 2011), especially in pets. The status of regional lymph nodes is a well-known prognosis factor in most malignant tumours in both humans and animals, holding a major impact in choosing the therapy (Nyman *et al.*, 2005; Servais *et al.*, 2011).

Pre-therapeutic staging includes assessment of the extent of the neoplasm through the lymphatic system. The most important lymph node in assessing lymphatic dissemination is the so-called “sentinel lymph node” (Sever *et al.*, 2012; Stan *et al.*, 2010; 2012). Regarding the link between mammary tumour and regional lymph nodes, the reaction of potential lymph node, is related to tumour topography and the direction of lymphatic drainage (Stan *et al.*, 2012; 2013; 2014).

In recent years, non-invasiveness has become a major goal in malignant diseases, since the imaging methods are continuously developing (Alam *et al.*, 2008; Liu *et al.*, 2014; Rubaltelli *et al.*, 2014; Stan *et al.*, 2014). However, lymph node biopsy is currently considered the main procedure to investigate the status of lymph nodes (Sever *et al.*, 2012). Considering that the accuracy of biopsy is only 70-85%, depending on the size of the fragments collected, and it is influenced by errors of sampling and sample processing, there is a tendency of finding an alternate, non-invasive investigating technique for staging malignant disease. The most suitable in this way are the imaging techniques.

This study is fully justified, both from theoretical (establishing the criteria for non-invasive investigation of lymph nodes) and practical considerations (compiling an ultrasonographic diagnostic algorithm), due to many similarities (hormonal status, estrogens-dependent breast tumours) between the lymphatic drainage of the mammary gland (Cassali *et al.*, 2014; Stan *et al.*, 2012) and also the variability of this system in both humans and carnivores.

MATERIALS AND METHODS

The draining lymph nodes of mammary glands belonging to the twelve dog females presenting tumours of the cranial and caudal abdominal mammary glands (A1 and A2, n=4), inguinal mammary gland (I, n=6) and cranial thoracic mammary gland (T1, n=2) were examined. The selection criteria are listed below:

Inclusion criteria

- Subjects with mammary gland tumours confirmed by clinical, imaging and histopathological examinations
- Stable general conditions at study beginning
- Owner consent

Exclusion criteria

- Subjects with surgically treated tumours
- Pregnancy
- Unstable general condition

In addition, eight healthy subjects were used as control group. All subjects underwent a clinical examination to highlight the potential associated comorbidities. The axillary and superficial inguinal lymph nodes were evaluated using an algorithm composed of gray-scale ultrasound, Doppler ultrasound, contrast-enhanced ultrasound (CEUS) and real-time elastography.

Gray-scale ultrasound of superficial lymph nodes was performed with Logiq 9® (General Electric) using a multifrequency linear probe (8-12 MHz). Doppler, elastographic and contrast enhanced ultrasound examination modes were available, in either real time or post processing. The following characteristics of the lymph nodes have been assessed: shape, size, (transverse and longitudinal axes), capsule delimitation from adjacent tissues, lymph node parenchyma, ecostructure and ecogenicity and hilum ecogenicity.

Doppler ultrasound pursued the following: the presence of vascular signal in lymph nodes, vascular type (normal vs. altered) and vascular pattern (peripheral vs. central).

For CEUS a second-generation contrast agent (SonoVue - Barraco-Italy) was used in two steps:

1. The suspension was prepared in its original packing. A quantity of 0.4ml SonoVue was injected in the peritumoral area in four distinct points, followed by gentle massage of the injected area. The progression of the contrast agent was followed on the device's screen up the first lymph node, noting the anatomic region where it was found. Also, the time of progression was recorded and the parenchyma's aspect has been assessed.

2. After placing a peripheral venous catheter, an average dose of 0.6ml SonoVue/animal (0.6ml SonoVue/10kg BW) from the freshly

prepared suspension was administrated on the main route of the catheter. On the secondary route, 5 ml of physiological serum was injected. When the contrast injection was done, the ultrasound timer started and the pattern of the lymph node parenchyma in the arterial and venous phases was recorded.

Real-time ultrasound elastography evaluated the region of interest (ROI) around the lymph nodes. The colour-coded signals used for the evaluation of the stiffness of tissues (blue-very hard; red-very soft; yellow-soft; green-intermediate) were registered. This data was stored as digital picture files, and subsequently analyzed by using Adobe Photoshop® to estimate the amount of each colour in the ROI numerically.

In all subjects, a sample for histopathological examination was taken. Lymph node ultrasound guided biopsy was performed in healthy subjects. Surgical excision of the sentinel lymph nodes during the mastectomy in subjects presenting

mammary tumours was performed and samples for histological examination were taken. Haematoxylin&Eosin staining was used.

RESULTS AND DISCUSSION

Forty-eight lymph nodes were evaluated. There have been recorded thirty- two lymph nodes in the malignant category, sixteen in the benign category.

Gray-scale ultrasound. Lymph node patterns were described according to Table 1, which shows the chosen parameters and corresponding scores for determining the lymph node pattern (malignant or benign). The gray-scale ultrasound characteristics used to differentiate between benign and malignant lymph nodes are illustrated in figure 1.

Doppler ultrasound used the following parameters: the presence of vascular signal in the lymph node, vascular type (normal vs altered), vascular pattern (central vs peripheral).

Tab. 1. Gray-scale ultrasound

Parameter	Characteristic	
	Benign	Malignant
S/L* ratio	< 0.5	>0.5
Echogenicity	Isoechoic/Hyperechoic	Hypoechoic
Echostructure	Homogenous	Inhomogeneous
Capsule features	Well defined with regular margins	Irregular
Hilum	Present, hyperechoic	Absent
Nr. of points	0	1

*S = short axis, L = long axis

Fig. 1. Grey scale US of normal (a) and pathological (b) lymph node. a) Oval shape, well defined capsule and homogenous echostructure of normal lymph node; b) Rounded shape, irregular appearance of capsule and inhomogeneous echostructure of pathological lymph node - arrows.

Each pattern has a score specified in Table 2 and illustrated in figure 2.

Using **contrast enhanced ultrasound (CEUS)** two patterns of enhancement in sentinel lymph nodes were observed: complete enhancement and partial enhancement with the presence of perfusion defects. Contrast-enhanced ultrasound performed after peritumoral administration of 0.4 ml SonoVue in four points revealed the presence of

hypoperfused focal areas of sentinel lymph nodes in eleven subjects from group I. After intravenous administration of contrast agent, the pattern of enhancement was analyzed in both groups. The results and score for each accomplished parameter are detailed in Table 3. The two pattern of enhancement are illustrated in figure 3.

On real time elastography, the colour-coded signals (blue-very hard, red-very soft, yellow-soft

Tab. 2. Color Doppler

Parameter	Characteristic	
	Benign	Malignant
Presence of hilar vascular signal	Present/Absent in lymph node < 2mm	Absent
Pattern	Ordered	Chaotic/Unordered
Distribution	Radially from the hilum toward the capsule	Peripheral, subcapsular
Nr. of points	0	1

Fig. 2. Doppler US of normal (a) and pathological (b) lymph node. a) The presence of hilar vascular signal in axillary lymph node. b) Chaotic arrangement with peripheral distribution of the vessels in a metastatic lymph node.

Tab. 3. CEUS

	Characteristic	
	Benign	Malignant
a) peritumoral adm		
Enhancement	Complete, intensive	Incomplete, with areas without contrast agent
Type	Homogenous	Inhomogeneous
Distribution	Centripetal	Aberrant
b) IV adm.		
Enhancement	Complete, intensive	Incomplete with filling defects or absent
Type	Homogenous	Inhomogeneous
Distribution	Centrifugal	Aberrant, distribution delays
Nr. of points	0	1

green-intermediate) were interpreted according to the Table 4.

The result of the gray scale ultrasound and Doppler examination were interpreted as follows:

Number of points less than 4: the nodes were interpreted as benign – (n=16 lymph nodes).

Number of points ranged between 4 and 6 : the nodes were interpreted as uncertain followed by CEUS and real-time elastography (n= 10 lymph nodes).

Number of points more than 6: the nodes were considered malignant (n=32 lymph nodes).

Nowadays the ultrasound exam is the method of first choice in the evaluation of adenopathies by conventional techniques, namely the grey-scale ultrasound and Doppler techniques (Stan 2010; Stan *et al.*, 2012), along with CEUS – Contrast-Enhanced Ultrasound (Stan and Badea 2012). In these conditions, the ultrasound techniques are the diagnostic methods whose values were

most often studied. These could indicate a change in the researcher's interest to use the non invasive techniques in lymph node assessment instead of conventional invasive methods. Gray scale ultrasound exam studies the diagnostic value of lymph node parenchyma ecogenicity, ecostructure, short to long axis ratio, hilar pattern and margins in both humans and animals (Dudea *et al.*, 2012; Whitman *et al.*, 2011). According to existing literature the two-dimensional ultrasound exam cannot make a real distinction between tumour-infiltrated lymph nodes (malignant ones) and inflammatory lymph nodes – which can be considered as benign lymph nodes (Dudea *et al.*, 2012; Nyman *et al.*, 2005; Stan *et al.*, 2010). The performances improved by using Doppler ultrasound in evaluating the lymph vascularisation, but not enough (Furukawa *et al.*, 2007; Stan *et al.*, 2010, 2013).

Contrast-enhanced ultrasound (CEUS), has added a new dimension to diagnostic possibilities,

Fig. 3. CEUS of superficial inguinal lymph node. a) Complete, homogenous enhancement in normal lymph node. b) Arterial phase showed several nonenhancing areas within the sentinel lymph nodes of tumoral mammary glands: a hypoperfused mass-asterisks, surrounded by relative normal enhancing of lymph node parenchyma – arrows.

Tab. 4. Real-time elastography

	Parameter	Characteristic
	Benign	Malignant
Stiffness, Quantified by the software in colour nuances	a) The whole lymph node is soft, mostly green, yellow or turquoise	>50% of lymph node is blue
	b) >50% of lymph node is green, with presence of small yellow or turquoise hyperechogenous nodules	>50% is blue except for some hyperechogenous nodules
	c) < 50% of lymph node is blue	All lymph node is blue and/or shares the color with surrounding tissue
		Hard lymph node colored blue on the edges, with necrotic areas – green/red
Nr of points		
		0
		1

by establishing an accurate diagnosis of focal lesions and trying to find its utility in assessing lymph nodes status (Goldberg *et al.*, 2004, 2005; 2011). By using the peritumoral injection of Sonaziod in 63 Sinclair swine with melanoma, Goldberg and al., (2011) stated the importance of this method in lymphatic mapping and its ability to localize the sentinel lymph nodes in cases of unpredictable drainage pathways. However, their results have shown that the differentiation between the benign and malignant lymph nodes was limited using only lymphosonography. Analysis of characteristic parameters of the method such as washing curves, transit times and quantifying the amount of contrast, improved the method, especially in describing the free lymph nodes (Stan *et al.*, 2014).

Another imaging technique is ultrasound elastography (real-time elastography), which quantifies the degree of tissue stiffness. Diagnostic methods based on this technique seem to be promising (Alam *et al.*, 2008; Das *et al.*, 2011; Lenghel *et al.*, 2012). In real-time ultrasound elastography, visualising the stiffness of the tissue requires transposition in colour mode, each colour signifying a level of stiffness. Thus, very hard tissues are represented by dark blue and intermediate shades of green, soft tissue are yellow and very soft ones are red. Evaluating this colours by using a special software, certain scores of elasticity can be calculated (Dudea *et al.*, 2012; Lenghel *et al.*, 2012). Based on four or five color-coded patterns of lymph nodes, an elasticity score

was proposed for the metastatic lymph nodes of the head and neck in squamos carcinoma. Alam *et al.*, (2008) established five elastographic patterns of lymph nodes based on the distribution and percentage of hard areas (or high elasticity) in lymph node parenchyma; pattern 1 being an absent or very small hard area to pattern 5, in which the hard area occupied the entire node. In this study the cut-off line between benign and malignant lymph nodes was set between pattern 2 and 3. Recently, Lenghel *et al.*, (2012) report a very good specificity and sensitivity in the differentiation between malignant and benign lymph nodes using the combined gray-scale appearance and elastographic images. The elastographic score that also includes the structural changes was based on eight patterns (Lenghel *et al.* 2012). Our study proposed an elasticity score of four patterns, trying to include the structural changes that indicated tumour infiltration too.

Currently, there is no standard of non-invasive lymph node staging in veterinary medicine. The current surgical approach by total excision of the lymph node without a prior investigation is an invasive procedure with potential risk of complications. It presents a high degree of intraoperative subjectivity and even errors in proper identification of the first lymphatic drainage station may occur. We consider that it is time to change the mainstream approach of tumour staging investigation in veterinary medicine with a non-invasive and less expensive alternative.

Fig. 4. Real time elastography of sentinel lymph nodes. a) Benign lymph nodes were mostly green; b) Hard lymph nodes revealed by the blue color present in all lymph node (the superficial inguinal lymph nodes) indicate the presence of metastatic infiltration.

CONCLUSIONS

The combination of the four ultrasound techniques used in the current study is a premiere. They were represented by: grey-scale ultrasound, Doppler ultrasound, contrast-enhanced ultrasound and real-time elastography. By using this algorithm, the metastatic infiltration of sentinel lymph nodes of mammary gland was assessed. These preliminary results are the first ones reported in veterinary medicine. Unfortunately, the statistical analysis is not relevant due to the low number of clinical cases, this being one of the major limitations of the proposed algorithm. Further studies are needed to confirm the accuracy of the diagnosis and to compare the results to those obtained by CT or MRI.

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The Institutional Bioethics Committee of University of Agricultural Science and Veterinary Medicine in accordance to Directive 2010/63 /EU of the European Parliament and of the Council on the protection of animals used for scientific purposes approved the study.