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ORIGINAL ARTICLE

Cell block sensitivity for immunohistochemical detection of cytokeratin 5, oestrogen and progesterone receptors in canine primary mammary carcinoma

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ABSTRACT. Mammary carcinomas are relatively common ailments among female canines aged around 10 years old, presenting an important morbidity with an average survival of five years. The cytoinclusion technique is frequently employed in human medicine as the investigative method of choice as it quickly provides resources for the determination of the correct therapeutic response, however, the effectiveness of the technique in canines remains understudied in veterinary medicine. This study aims at evaluating the degree of correlation with immunohistochemical marking for cytokeratin 5 (CK5), oestrogen and progesterone receptors (ER and PR) between the cytoinclusion and the histopathology technique in mammary carcinomas. Twenty-five samples of mammary carcinoma, both for the cytoinclusion and histopathological techniques were submitted for histological processing; microscope slides were created for hematoxylin-eosin (HE) staining and the immunohistochemical technique (IHC) was assessed for the ER, PR and CK5 receptors. Through the HE staining, we reached a concordance rate of 100% between the cytoinclusion and the histopathological analysis in the diagnosis of carcinomas. The immunohistochemical assay presented sensitivity of 85.71%, 95.45% and 100% and Cohen's kappa of 0.78, 0.84 and 0.95 for ER, PR and CK5, respectively, as well as 100% specificity and $P<0.01$ for all three markers. Therefore, cytoinclusion represents an accessible, fast and low-cost method, offering high sensitivity for the cytomorphological and immunohistochemical diagnosis of mammary carcinoma in female canines.

Key words: cytoinclusion, histopathological, immunohistochemistry, tumor.

RESUMEN. Los carcinomas mamarios son relativamente comunes en hembras caninas con edades de alrededor 10 años y representan un importante factor de morbilidad para estas hembras; la técnica de citoinclusión es frecuentemente empleada en medicina humana como un método de diagnóstico que permite proveer información rápida para la determinación de una conducta terapéutica correcta, sin embargo, la efectividad de esta técnica permanece poco estudiada en medicina veterinaria. El objetivo del estudio fue evaluar el grado de correlación entre marcadores inmunohistoquímicos para citokeratina 5 (CK5), receptores de estrógenos y progesterona (RE y RP) entre la técnica de citoinclusión y la técnica histopatológica en carcinoma mamario. Veinticinco muestras de carcinoma mamario para la técnica de citoinclusión e histopatológica fueron sometidas a procesamiento histológico, láminas fueron confeccionadas para la tinción mediante hematoxilina y eosina (HE), y la técnica de inmunohistoquímica para identificar la expresión de RE, RP y CK5. Mediante la tinción de la HE fue encontrado una proporción de concordancia del 100% entre el análisis de citoinclusión e histopatológico de carcinoma mamario. El análisis inmunohistoquímico evidenció una sensibilidad del 85,71%, 95,45% y 100% y un índice kappa de Cohen de 0,78, 0,84 y 0,95 para RE, RP y CK5, respectivamente, así como 100% de especificidad $P<0,01$ en todos los marcadores. De esta forma, la citoinclusión representa un método rápido, accesible y de bajo costo, que ofrece alta sensibilidad para el diagnóstico citomorfológico e inmunohistoquímico en carcinoma mamario de perras.

Palabras claves: citoinclusión, histopatológico, immunohistoquímica, tumor.

INTRODUCTION

The incidence of mammary neoplasms in female canines and women has been increasing exponentially throughout the world, representing about 20% of all new cases. Human breast cancer represents 25% of all neoplasms afflicting humans and mammary tumors have the highest incidence among the neoplasms afflicting female canines, representing 50% of all neoplasms, with 45% to 50% of those tumors being malignant (Misdorp *et al* 1999, Cassali, 2011, Siegel *et al* 2012, Terra *et al* 2012, Inca 2011).

The high incidence and the aggressiveness of this disease demanded search for fast, low-cost and less invasive diagnostic methods (Zanoni *et al* 2013). In this context, the cytology technique has earned a position in the spotlight for offering diagnostic resources in cytomorphological studies, immunohistochemical studies, flow cytometry and molecular biology techniques. It aids in the diagnosis of benignant and malignant ailments in several organs (Zuccari *et al* 2001, Bueno *et al* 2013, Ferreira *et al* 2013), as well as in determining the need of anaesthetising the animal (Zuccari *et al* 2001).

It is worth mentioning that diagnostic methods widely employed in Human Medicine are still seldom explored in Veterinary Medicine, especially regarding the application of cytoinclusion, or cell block (CB), without the use of agarose (Bueno *et al* 2013). This method was originally proposed for processing electronic microscopy samples (Mulder *et al* 1997, Sanchez and Selvaggi 2006) and may potentially optimize the cytological sample by enabling its "blocking" in paraffin for posterior processing, as happens with a histological sample (Zanoni *et al* 2012).

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This presents several advantages, such as an architectural analysis similar to histopathological examinations (Constantian and Girolami 1973) and molecular tests (Crapanzano *et al* 2014).

The use of the cell block technique to determine the expression of oestrogen (ER), progesterone (PR) and cytokeratin 5 (CK5) receptors allows a better therapeutic approach (Bueno y col 2013, Zanoni *et al* 2012; Horta *et al* 2012). An increased expression for the oestrogen (ER) and progesterone (PR) receptors is related to a better prognosis while the positivity of the CK5 receptor is related to a worse prognosis, in both canines and humans with mammary carcinomas (Terra *et al* 2012, Horta *et al* 2012, Figueiroa *et al* 2012). In canines, the expression of ER is higher in benignant tumors than in malignant ones and is also higher in complex and mixed tumors in comparison with simple sub-types (De Las Mulas *et al* 2004).

The expression of PR is mediated by ER, and its positivity is considered as a marker for working oestrogen receptors. Around 50% of all tumors positive for ER are also positive for PR and, among these, around 75% are sensitive to hormonal therapy. Tumors that are positive for ER and negative for PR are less sensitive to hormonal therapy, suggesting a possible contribution of PR in the response to this treatment. Tumors that are negative for ER and positive for PR have shown an intermediary response to hormonal therapy (Yamashita *et al* 2006, Zuccari *et al* 2008).

Myoepithelial cells express cytokeratins specific for base cells, such as CK5, which is part of the cytoskeleton of these cells. Located in the basal and parabasal layers, as well as in mammary progenitor cells, which have the ability to differentiate in glandular and myoepithelial cells, the expression of the CK5 gene is related to a worse prognosis both in canines and humans. In the latter, it is related to the basal phenotype, which has the worst prognosis (Figueiroa *et al* 2012, Matos *et al* 2005).

This study aims at determining the degree of correlation between the immunohistochemical marking for the ER, PR and CK5 receptors in the cytoinclusion and histopathological techniques in the diagnosis of canine mammary carcinoma, as well as in diagnoses by cytoinclusion and in the morphological diagnosis of the surgical specimen in order to verify the sensitivity of the method.

MATERIAL AND METHODS

SAMPLES

Samples were collected from 25 female dogs that were at least 5 years old, without bias for any breeds. These dogs presented a definitive diagnosis of mammary carcinoma and their owners sought treatment in the Veterinary Hospital of the College for Veterinary Medicine and Animal Sciences (FMVZ-*Faculdade de Medicina Veterinária e Zootecnia*) of the São Paulo State University (UNESP), Botucatu campus.

The Brazilian Ethics Commission in the Use of Animals (protocol no. 119/2014-ECUA) approved the study and the owners of all animals involved in the study signed a Free and Clarified Consent Term authorising the collection of material and the use of the data in research papers.

Cytological, histopathological and immunohistochemical studies were conducted in the Veterinary Pathology Laboratory, in collaboration with the Pathology Laboratory of the Botucatu Medicine College-UNESP.

CELL BLOCK TECHNIQUE AND MORPHOLOGICAL ANALYSIS

The animals underwent radical or unilateral mastectomy, as well as hysterectomy, salpingectomy and oophorectomy. After surgical removal, the cell block technique was conducted in a fresh piece. According to the pilot test conducted, there were no differences in the quality of the material obtained regarding size of the dog and before or after surgery.

According on the size of the wound, the piece was punctured with a 22 to 26 gauge needle in a 10 mL BD syringe (Cameco, London, UK) connected to the valeri cytology-aspirator® (MPJ *Equipamentos Médicos*) until a minimum amount of 0.1 mL was obtained per animal. In each case, the samples were dried at room temperature and then fixed in 100% methanol for later application of the rapid Romanowsky stain (Diffi Quick) and Giemsa stain (Raskin and Meyer 2010).

For the cell block, the material in the needle and syringe was initially fixed in 2 mL of 95% alcohol for 30 seconds for cohesion, and then by the aspiration of 8.0 mL of 7.4% buffered formalin solution. The sample remained inside the syringe for fixation during 24 hours. After the material was removed with the aid of anatomical tweezers, it remained for 30 minutes in alcoholic-eosin and then underwent histotechnical processing and staining through the Hematoxylin-Eosin (HE) technique, i.e. inclusion in a paraffin cassette, 4 μm microtomy and staining battery.

The cytomorphological analysis through HE staining was conducted independently by two veterinary pathologists in an optical microscope (Zeiss Axio Lab.A1). For each slide, 10 fields were read with 400x magnification. The neoplasms were classified according to the criteria established by Zakhour and Wells (1999) and Raskin and Meyer (2010) regarding cellular composition. Inter-observer variation was resolved by simultaneous dual re-evaluation.

The smears, the cytoinclusion slides and the surgical piece slides were analysed separately, so that the pathologists could not identify the corresponding slides.

HISTOPATHOLOGICAL CLASSIFICATION

In each case, samples were collected and fixed in 10% neutral buffered formalin solution. Tissue samples were

processed routinely, embedded in paraffin wax and sectioned for Hematoxylin-eosin (HE) staining. Tumor classification was defined according to the WHO classification (Misdorp *et al* 1999) and the grading systems for canine mammary tumors (Goldschmidt *et al* 2011).

The criteria of malignancy was based in the presence of cells with enlarged nuclei and prominent nucleoli, according with: tumor type, significant nuclear and cellular pleomorphism, mitotic index, presence of randomly distributed areas of necrosis within the neoplasm, peritumoral and lymphatic invasion, and regional lymph node metastasis (Goldschmidt *et al* 2011).

Tumor sections were examined in an optical microscope (Zeiss Axio Lab.A1) by two veterinary pathologists. For each slide, 10 fields were read with 400x magnification. Inter-observer variation was resolved by simultaneous dual re-evaluation.

IMMUNOHISTOCHEMICAL STUDY

Serial sections 4 µm thick were cut from each block and placed in Slidetech Plus Novocastra® Newcastle UK glass slides. After deparaffinisation in xylene, the slides were rehydrated and washed in Tris Buffered Saline (TBS). The endogenous peroxidase activity was quenched by a 10-minute incubation in a mixture of 3% hydrogen peroxide solution in 100% methanol (Sigma). The antigen retrieval was conducted with citrate buffer (pH 6.0) in a pascal pressure cooker. Slides were cleared with TBS and placed at room temperature for 1 hour. They were then incubated with antibodies for ER (LH2 clone, dilution 1:40, Novocastra, UK), PR (SP2 clone, dilution 1:50, Neomarkers, USA) and CK5 (clone XM26, dilution 1:50, Neomarkers, USA) overnight at +8°C. The signal was visualised using the DAKO-EnVision Kit (k 5007 HRP Rabbit/Mouse) according to the instructions of the manufacturer. After washing with TBS, slides were kept in diaminobenzidine tetrahydrochloride for 7 min then counter-stained with Mayers hematoxylin.

We conducted a comparative analysis for the ER and CK5 markings in the cytoinclusion slides and in the surgical piece slides. We considered positive cases those in which ER and PR presented nuclear marking exceeding 1% (Hammond *et al* 2010), and those in which the cytoplasmic marking of CK5 presented immunopositivity exceeding 10% (Ramalho *et al* 2006).

For CK5, we considered positive cases with two crosses (2+) when, at most, 50% of the neoplastic cells presented marking and positive cases with three crosses (3+) when more than 50% of the neoplastic cells presented marking (Ramalho *et al* 2006).

The negative control was conducted for all antibodies by omitting the antibody and as positive control we employed internal and external controls, as parameters for the reactions, with the internal control represented by the non-tumoral mammary glands in the surgical pieces.

The validity of the CB method through immunohistochemical marking was determined through the assessment of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). For ER and PR, cases with marking in more than 1% of the neoplastic cells were considered positive, while for CK5, markings in more than 10% of the neoplastic cells were considered positive, as observed in table 1.

STATISTICAL ANALYSIS

The concordance between the histopathological assay, the cytoinclusion and immunohistochemical techniques was assessed by weighted Cohen's Kappa as follows: values <0 suggest no concordance; values between 0-0.20 suggest a slight concordance; 0.21-0.40 suggest reasonable concordance; 0.41-0.60 moderate concordance; 0.61-0.80 substantial concordance; 0.81-1 nearly perfect concordance. Statistical difference was considered when $P \leq 0.05$. The statistical analysis was conducted using the software IBM SPSS 20.

RESULTS

A total of 25 samples of mammary carcinoma were analysed in this study from animals aged between 5 and 15, averaging 9.92 years old. Small, medium and large size animals represented 48%, 32% and 20% of the cases respectively, with an average weight of 10 kg. The average development period of the tumors was 11.32 months, with an average deviation of 7.28.

HISTOPATHOLOGICAL AND CYTOINCLUSION CLASSIFICATION

Regarding the histopathological classification, 48% were carcinomas with mixed tumors, 28% were tubulopapillary carcinomas and 24% were complex carcinomas.

There was a high correlation between the morphology of the surgical piece (figure 1) and the cytomorphology of the cytoinclusion assay (figure 2), with 100% concordance

Table 1. Samples considered positive in the immunohistochemical marking in neoplastic cells above 1% for RE and RP and above 10% for CK5.

	Cytoinclusion (%)	Surgical Piece (%)
RE	24	24
RP	84	88
CK5	92	92
	++40	++48
	+++52	+++44

*++: when up to 50% of the neoplastic cells presented marking; +++: when over 50% of the neoplastic cells presented marking.

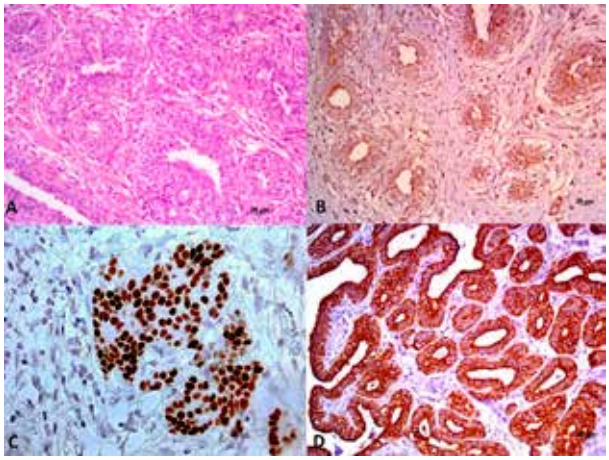

Figure 1. Sample from the surgical piece taken from Animal 3, stained through HE (A), positive marking for RE (B), positive marking for RP (C) and (+++) marking for CK5 (D). Obj. 20X, bar 20μm.

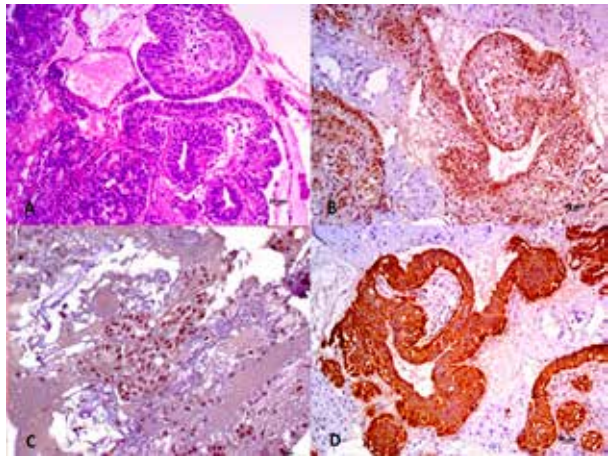

Figure 2. Sample from the cytoinclusion assay taken from Animal 3, stained through HE (A), positive marking for RE (B), positive marking for RP (C), positive (+++) marking for CK5 (D). Obj. 20x, bar 20μm.

between the histopathological assay and the cytoinclusion technique. The same is true for the positivity found in both methods for the oestrogen, progesterone and cytokeratin 5 receptors.

The CB marking for ER presented sensitivity of 85.71% and specificity of 100%, 100% PPV and 94.74% NPV. The accuracy was 96% with a Kappa index of 0.78 and $P<0.01$.

PR presented a sensitivity of 95.45%, specificity of 100%, 100% PPV and 75% NPV. The accuracy was 96% with a Kappa index of 0.84 and $P<0.01$.

The CK5 analysis presented 100% sensitivity, specificity, PPV, NPV and accuracy, with a Kappa index of 0.95 and $P<0.01$.

Finally, in one case (sample 08) the marking was positive for ER in the cytoinclusion assay and negative in the surgical piece assay. In another (sample 25), the marking was considered negative for both ER and PR in the cytoinclusion assay. In two cases (samples 10 and 21), CK5 was rated 3+ in the cytoinclusion assay and 2+ in the surgical piece assay.

DISCUSSION

Cytoinclusion has been used in human medicine for over 50 years, gaining strength towards the end of the 1990s and beginning of the 2000s due to the simplification of the technique, which no longer required the use of agarose (Bueno *et al* 2013). The method is reliable and may be used for screening patients, assessing predictive factors and prognoses related to these markers, as well as presenting reduced costs due to requiring fewer materials.

In this study, the technique provided enough material for conducting smear and cytoinclusion assays, enabling the diagnosis of the neoplasm and the research regarding the oestrogen, progesterone and cytokeratin 5 receptors, confirming the ease and quickness this technique presents in the collection of material.

As happens in human females, the verification of positivity for immunohistochemical markers enables the application of neoadjuvant chemotherapy with an adequate choice of chemotherapeutic agents, which makes subsequent surgical procedures less complicated, reducing costs and improving the prognosis (Nguyen *et al* 2011).

The high correlation between the morphology of the surgical piece and the cytomorphology of the cytoinclusion assay, together with the positivity found in cytoinclusions conducted in this study for the oestrogen, progesterone and cytokeratin 5 receptors, is in accordance with the percentages found for surgical pieces in the literature regarding mammary tumors (Figueiroa *et al* 2012). The results are also similar to those reached by Bueno *et al* (2013) in a comparative study between cytoinclusion and surgical specimen of mammary tumors in women. According Fowler and Lachar (2008), who describes the quality of immunostaining in the cell block sections, it is identical to that of paraffin embedded tissue fixed in formalin.

The literature regarding the use of cytoinclusion in veterinary medicine is scarce. Zanoni *et al* (2012), in a comparative study between the cell block technique in agarose and smears of canine mammary tumors, found diagnostic concordance levels of 13.7% and 5.9%, respectively, in comparison with the surgical piece, showing a better representativeness of the CB technique.

Paiva *et al* (2011[a]) compared the cytological assay and cytoinclusion in 12 neoplasms, reaching concordance in 58.3% of the cases. In all cases, an immunohistochemical assay for cytokeratin and vimentin confirmed the histological origin.

In this study, the high degree of correlation between the surgical specimen and the cytoinclusion technique

is in accordance with the figures found by Bueno *et al* (2013), whose samples obtained by cytoinclusion provide a material with high cellularity and representativeness of the wound, enabling the correlation between techniques.

Furthermore, the prevalence of carcinoma-mixed type, tubulopapillary carcinoma and carcinoma-complex type in our results is in accordance with other studies in mammary female dogs that describe carcinomas and malignant mixed tumors as the most frequent tumors, in addition to benign cases. However, these numbers have considerably variations due to the different methods of classifying tumors, especially the separation of mixed tumors and carcinomas (Else and Hannant 1979, Moulton 1990). Comparing with recent studies in female dogs, the prevalence of papillary carcinoma was observed by Kamiguchi *et al* (2016).

A recent consensus issued by the *College of American Pathologists,* the *International Association for the Study of Lung Cancer and the Association for Molecular Pathology* recommends the cytoinclusion technique for EGFR immunohistochemical assay for lung cancer (Lindeman *et al* 2013).

The use of IHC during the cytoinclusion technique has shown better results in comparison to smears, presenting a lower amount of artifacts and false-positives, as well as a good correlation with the findings in the surgical specimen (Bueno *et al* 2013).

In cases with differences in the markings, it is important to consider the heterogeneous morphology of mammary tumors. A single sample often presents areas with varying appearances, hyperplastic sites parallel to neoplastic nodules and mixed-pattern areas (Zanoni *et al* 2013). Therefore, it is possible that the marking and score differences of some samples are caused by this situation.

Another aspect to be considered is the presence of necrosis in the cytological sample, which interferes both with the diagnosis of the neoplasm and with the immunohistochemical assays (Paiva *et al* 2011[b]), as observed in a false negative case for the ER and PR markers. This highlights the importance of understanding the technique during the collection of samples.

Therefore, based on the results reached by this study, we suggest the use of this diagnostic technique in the practice of Veterinary Medicine. The high sensitivity found for the immunohistochemical marking of ER, PR and CK5, in addition to the high correlation between the cytomorphological diagnosis through cytoinclusion and the morphological diagnosis through the surgical specimen, makes it plausible to state that cytoinclusion is as efficient in veterinary medicine as in human medicine. Thus, it represents a safe and low-cost method for the study of prognostic markers with CB.

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