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EVALUATION OF THE CELL BLOCK TECHNIQUE FOR THE DIAGNOSIS OF MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL CANINE APPENDICULAR OSTEOSARCOMA

AVALIAÇÃO DA TÉCNICA DO BLOCO CELULAR PARA O DIAGNÓSTICO DO OSTEOSSARCOMA APENDICULAR CANINO MORFOLÓGICO E IMUNOHISTOQUÍMICO

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ABSTRACT

Canine osteosarcoma (OSA) is the most common primary bone tumor in dogs worldwide, accounting for up to 85% of all malignant bone tumors. The cell block technique involves the insertion of cytological specimens into paraffin blocks to aid in the quality of the cytological diagnosis and to obtain reserve specimens for use in other techniques, including immunohistochemistry. This study aimed to evaluate the sensitivity and specificity of the cell block technique for the diagnosis of canine appendicular OSA. Eleven dogs ranging from 8 to 13 years old with presumed OSA diagnosis were submitted for bone aspirate cytology, cell block preparation, and histopathological evaluation. Bone cytology was performed for cell block preparation. The aspirated material was fixed with 1 mL alcohol at 95% for five minutes in the syringe itself, followed by aspiration of 9 mL of 10% formalin for 24 hours. The syringe was maintained in an upward position for cell decantation. The material was then routinely embedded in paraffin. Optical microscopic analysis was performed for cytological, cell block, and histopathological slides by three independent pathologists. Additionally, immunohistochemistry (IHC) was performed on samples from the cell block and histopathological evaluation. There was concordance of the cell block with

the histopathology and immunohistochemical labeling of positive osteopontin with high sensitivity and specificity. The cell block technique was effective for OSA diagnosis and had high reproducibility in IHC staining.
Keywords: Bone, cell block, immunohistochemistry, histopathology, cytopathology.

RESUMO

O osteossarcoma canino (OS) é o tumor ósseo mais comum nos cães, responsável por até 85% dos tumores ósseos malignos. A técnica de *cell block* envolve a inserção de amostras citológicas em blocos de parafina, auxiliando na qualidade do diagnóstico citológico e na obtenção de amostras de reserva para uso em outras técnicas, incluindo imuno-histoquímica. Esta pesquisa teve como objetivo avaliar a sensibilidade e especificidade da técnica de citoinclusão no diagnóstico de OS apendicular canino. Onze cães, de 8 a 13 anos de idade com diagnóstico presumido de OS foram submetidos à citologia aspirativa óssea, bloqueio celular e avaliação histopatológica. Para o bloqueio celular, foi realizada citologia óssea e o material aspirado foi fixado com 1mL de álcool a 95%, por cinco minutos na própria seringa e, em seguida, 9 mL de formalina a 10% foram aspirados por 24 horas, mantendo a seringa posicionada para cima para decantação celular. Em seguida, o material era rotineiramente incorporado em parafina. A análise microscópica óptica foi realizada para lâminas citológicas e histopatológicas por três patologistas independentes. Além disso, a imuno-histoquímica (IHC) foi realizada nas amostras em citoinclusão para avaliar marcadores diagnósticos. Houve concordância do bloqueio celular com a histopatologia e marcação imuno-histoquímica do osteopontina positivo com alta sensibilidade e especificidade. Foi possível inferir que a técnica de bloqueio celular era eficaz para o diagnóstico de OS e apresentava alta reprodutibilidade na marcação por IHC.
Palavras-chave: Osso, inclusão celular, imuno-histoquímica, histopatologia, citologia.

INTRODUCTION

Osteosarcoma (OSA) is the most common bone tumor in dogs; this type of tumor can also affect other species such as humans, cats, and other domestic animals (MORRIS and DOBSON, 2007; KARNIK et al., 2012). It is a highly aggressive and locally invasive tumor, becomes progressively more painful as it grows, promotes bone destruction and spreading via the bloodstream, and usually metastasizes to the lungs (OLIVEIRA and SILVEIRA, 2008; KRAJARNG et al., 2012).

The most important OSA differential diagnoses include osteomyelitis, secondary bone tumors, and chronic bone fractures. The diagnosis is based on clinical history, detailed physical examination, imaging tests, cytological examination, biopsy, and subsequent histopathological evaluation (RYSEFF and BOHN, 2012). Cytology is a reliable and minimally invasive diagnostic method and is increasingly being used in veterinary medicine with a diagnostic tool that has been expanding over the years (FIELDER and MAHAFFEY, 2009). However, given that cytology evaluates cells, the tissue architecture is lost (FIELDER and MAHAFFEY, 2009).

The cell block technique or cytoinclusion involves the insertion of cytological specimens into paraffin blocks to aid in the quality of the cytological diagnosis and to obtain reserve specimens for use in complementary diagnostic techniques, such as immunohistochemistry (VARSEGI and SHIGHAM, 2009). This technique is still an unexplored approach in veterinary medicine but has advantages such as reduced cost (compared with histopathological examination), low invasiveness, and rapid outcome. It allows the identification of histological patterns, thus decreasing the number of cases with an indefinite diagnosis (BRIFFOD et al., 2000). This study aimed to evaluate the diagnostic efficacy of the cell block technique in dogs with appendicular OSA.

MATERIAL AND METHODS

This prospective study included dogs with primary appendicular OSAs from January 2015 to January 2016 from the Veterinary Teaching Hospital at UNESP, Botucatu, Brazil. All cytological samples were collected from patients prior to the surgical procedure. All procedures were in accordance with the national and international ethical guidelines for the use of animals in research and were approved by the ethics committee of UNESP Botucatu (protocol number: 48/2015). Twenty-three dogs with appendicular OSA were included in this study.

2.1 Aspiration Cytopathology

Fine needle aspiration (FNA) cytology consisted of 30 × 8 mm disposable hypodermic needles, 10 mL disposable syringes, Valeri cytoaspirator (FIGURE 1), 26 × 76 mm histological slides with smooth and frosted ends, and slide and pencil containers to identify blades.

After assembling the cytoaspirator, syringe, and needle system, the examination was performed as follows: application of iodinated alcohol on the skin, introduction of the needle into the lesion, and promotion of negative pressure by pulling the entire cytoaspirator trigger to retract the syringe plunger. Patient sedation or anesthesia was not performed prior to the procedure. As a result, the vacuum formation in the system made it possible to aspirate the cellular material into the needle.

The needle was moved in several fan-like directions without removing it from the lump, thus preventing air from penetrating and maintaining negative pressure. Once this was done, the needle was removed from the lump.

To form a small cell button on a microscope slide, the material was deposited in a single location to obtain a good quality smear (THRALL, 2000). To perform the smears, the cytological material was quickly distended on a cytological slide, and a new cytological slide was placed perpendicularly on the material deposited in the first slide by using a single rapid movement. The top slide was displaced in opposite directions to obtain a cell smear. Giemsa staining was then performed for cell evaluation.

2.2. Cytoinclusion Technique

After aspiration in the mass with the plunger at rest, a procedure similar to that of aspiration cytology was performed: 1 mL of 95% alcohol was aspirated using the same material (a cytoaspirator, 10 mL syringe, and 30 × 8 needle) and the same technique, and it was allowed to stand for five minutes to fix the material. Approximately 9 mL of formol was then aspirated in the same syringe with the objective of achieving maximum preservation of the cells obtained in the procedure. Thereafter, 10% was aspirated and fixed for another 24 hours while keeping the syringe positioned upward for cell decantation (Figures 1A and 1B).

Figure 1: A: Photomicrograph showing aspiration puncture in the right humerus distal region. B: Syringe obtaining aspirated material immersed in the fixative solution.

After 24 hours, the needle was removed to expel the harvested material and then inserted into the histological cassettes. In this study, it was necessary to use histological sponges (Leica Biosystems, Wetzlar, Alemanha) in the cassettes to avoid material loss (Figure 3).

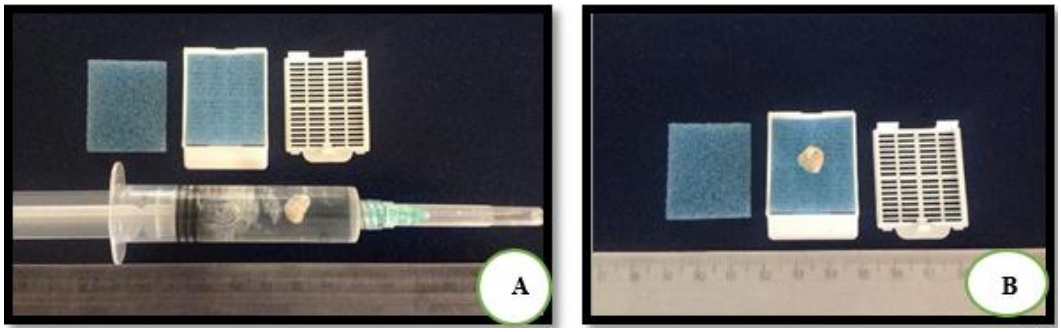

FIGURE 3: Photomicrograph of cytoinclusion procedures. A: Syringe containing material aspirated from OSA. B: Material obtained by FNA from the syringe after fixation in 95% alcohol and buffered formalin. It is important to use a small sponge to avoid the loss of material.

The material obtained was subjected to increasing alcohol baths, followed by xylol and paraffin baths that each last a maximum of 20 minutes. The samples were wrapped in paraffin, and 4 µm sections were stained with hematoxylin and eosin (HE) and immunoassays for cytoinclusion.

The number of slides per case varied. The following were evaluated in each slide: cellularity, nucleus/cytoplasm ratio, nuclear size variations, chromatin distribution pattern, nucleolus number and nucleolar morphology, atypical mitoses, cohesion, and pattern of spatial organization between cells.

2.3 Histopathological Processing of the Sample

After macroscopic examination, tumor fragments from the surgical amputation were fixed in 10% buffered formalin, decalcified in 10% nitric acid, and then processed using the usual histological techniques of dehydration, diaphanization, and paraffin inclusion. Thereafter, they were cut to a maximum thickness of 4 µm and stained with HE for light microscopy.

2.4 Histopathological Classification

The sections of neoplastic bone tissue that were stained with HE were examined under an optical microscope to allow the characterization of the morbid process. The process was classified according to the system described by Meuten (2002) on the basis of the evaluation of two observers (AH and NSR).

2.5 Sample Processing: Immunohistochemistry

To perform immunohistochemistry, paraffin blocks were used from the cytoinclusion. The paraffin blocks were cut into a 3 µm microtome and distended in histological slides prepared with 3-aminopropyltrimethoxy silane (Sigma A3648). The aim of this procedure is to promote a greater adhesion of the cuts in the glass slides to avoid loss of material during immunohistochemical processing.

For the technique, osteopontin, and positive control, adjacent osteoblasts from the tumor samples that were collected postsurgical amputation were used as positive internal control. Negative controls were prepared by replacing the primary antibody with TRIS buffer solution. After antigen recovery in citrate (pH 6.0) in a pressure cooker (Pascal), endogenous peroxidase blockade was performed in 3% H_2O_2 diluted in methanol. Protein blocking was performed using 6% Molico milk. All incubations of the primary antibody were performed at 4 °C overnight with Osteopontin was diluted at a ratio of 1:50. HRP antibody secondary antibody (EnVision) was applied for one hour and DAB for 5 minutes.

2.6 Reading and Interpretation of the Sample

With the aid of an optical microscope, the slides were evaluated with a 40-fold objective. In cytology and cytoinclusion, the identification of sufficient numbers of cells with preserved morphology was the criterion used for the sample to be considered viable (the number was at least 100 cells). The samples were scored as positive or

negative. Positive staining was considered when at least 10% of the osteoblasts were positive (brown staining in the cytoplasm), and negative staining was considered when more than 90% of the cells were negative (blue staining).

2.7 Statistical Analysis

The data obtained were evaluated using 2 × 2 contingency tables (BERQUÓ, 2001), and the sensitivity and specificity of the methods were calculated as follows:

Sensibility: Detect the truly positive

$$Sensibility\ (\%) = \frac{\text{true positive (PV)}}{\underset{false\ negative\ (FN)}{\text{true positive (PV)+}}} \times 100,$$

Specificity: Detect the truly negative

$$\text{Specificity}(\%) = \frac{\text{true negative } (TN)}{\underset{true\ negative\ (TN)}{false\ positive\ (FP)\ +}} \times 100.$$

RESULTS

The cytological and tissue specimens of the 11 animals included in the study were processed. Furthermore, smears, cytoinclusion samples, and histological sections were obtained. Table 1 presents the samples of primary OSAs from dogs of different breeds, ages, and locations.

Table 1: Relationship of age, race, sex and location of lesions in dogs submitted to FNA cytology, cell block technique (cytoinclusion), and histopathology.

ANIMALS	SEX	AGE	RACE	LOCATION
A1	Female	11	Pit Bull (medium)	Scapular region, right thoracic member
A2	Female	11	Golden Retriever (large)	Distal, right femur
A3	Female	8	German Shepherd (giant)	Proximal, humerus right
A4	Male	12	Akita (large)	Region coxofemoral
A5	Female	12	Siberian Husky (medium)	Distal, right femur
A6	Female	12	SRD (medium)	Radius and ulna, distal right
A7	Male	9	Rottweiler (large)	Radius and ulna, distal right
A8	Male	13	Rottweiler (large)	Right metacarpals
A9	Male	11	Pit Bull (medium)	Proximal, right humerus
A10	Male	13	English Setter (large)	Distal, right femur
A11	Female	10	Pinscher (small)	Distal, right femur

There were more females (6/11 animals) than males; however, the difference was not significant. The age of the animals ranged from 8 to 13 years, with a mean age of 11 years (Table 1).

The lesions were more frequent in the distal region of the right femur (four animals affected [36.37%]), followed by the right proximal humerus region and right distal radius and ulna (two animals affected in each region [18.18%]). The remaining specimens were located in the right scapular region, femur, and metacarpal (one animal affected [9.09%]) (Table 2).

In relation to the size of the affected animals, medium- and large-sized animals were predominantly affected by lesions, among which the Pit Bull and Rottweiler breeds were the most affected (two animals each [18.18%]). In turn, the other breeds were cited only once. Among the small dogs, only the Pinscher was affected by the disease.

Regarding the cytoinclusion technique, there was no difficulty during harvesting to the point of needing sedation.

On the basis of the histopathological pattern described by Meuten (2002), nine cases of osteoblastic OSA (82%) and two cases of fibroblast OSA (18%) were found (Table 2).

Table 2: Neoplastic classification of OSA in dogs with aspiration cytology, cytoinclusion, and histopathology.

Animals	Cytopathology	Cell Block	Histopathology
A1	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A2	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A3	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A4	OSA Fibroblastic	OSA Fibroblastic	OSA Fibroblastic
A5	OSA Fibroblastic	OSA Fibroblastic	OSA Fibroblastic
A6	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A7	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A8	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A9	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A10	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic
A11	OSA Osteoblastic	OSA Osteoblastic	OSA Osteoblastic

OSA: Osteosarcoma

Osteoblast islands were identified. They were surrounded by tumor cells with a variable degree of cellular pleomorphism; could have multinucleated giant cells, isolated cells, or agglomerated cells; and resembled osteoblasts. This corroborated the findings of Fielder and Mahaffey (2009). For fibroblastic OSA, the presence of spindle tumor cells with extensive areas of osteoid matrix involving clusters of tumor cells is a defining characteristic of the tumor classification of OSA defined by Meuten (2002).

Figures 3A to 3F show the descriptive results of the cytopathological, cytoinclusion, histopathological, and immunohistochemical examinations of osteoblastic OSA.

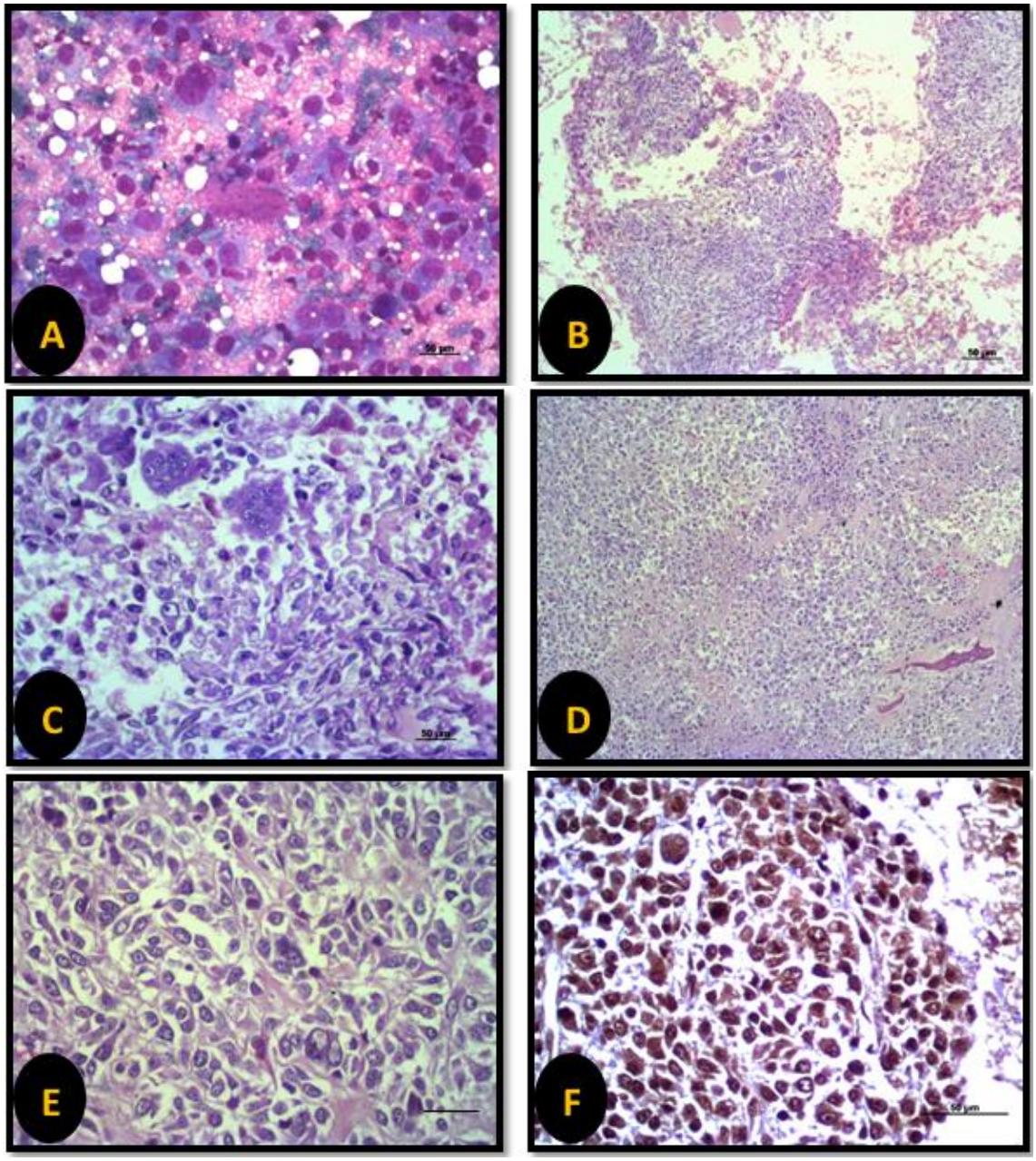

Figure 3: Photomicrograph of cytology, cytoinclusion, and histology of canine OSA. (A) Aspiration cytology: Malignant mesenchymal cells with morphological characteristics of neoplastic osteoblasts, adjacent bone matrix, multinucleated cells (Giemsa stain, obj. 40x). (B) Low-magnification image of an OSA cytological evaluation. It is possible to observe a neoplastic osteoblast and a high number of cells for evaluation (10x). (C) Cytoinclusion high cellularity of malignant osteoblasts. Preservation architectural pattern of neoplasia, morphological characteristics of neoplastic osteoblasts, adjacent bone matrix, multinucleated cells, and micronucleus (HE, obj. 40x). (D) Histopathological: High cellularity of malignant osteoblasts and preexisting bone trabeculae with a large proliferation of neoplastic osteoblasts and nonmineralized bone matrix production (HE, obj. 10x). (E) Histopathological: (obj. 400x). (F) Positive immunohistochemistry for osteopontin antibody in canine OSA cytoinclusion (IHQ, obj. 40x).

Figures 4A to 4F show the descriptive results of the cytopathological, cytoinclusion, histopathological, and immunohistochemical examinations of fibroblast OSA.

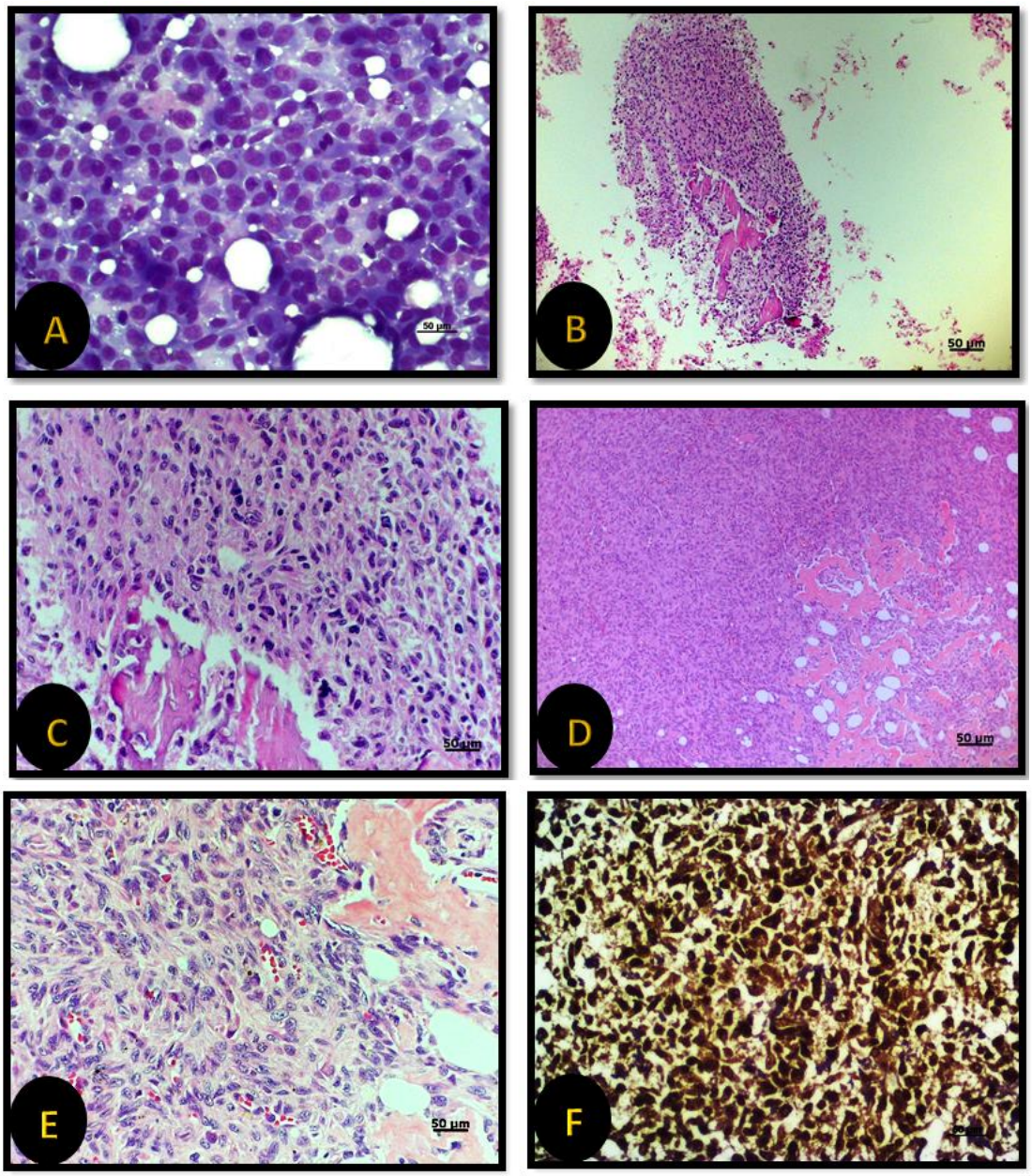

Figure 4: Photomicrograph of canine OSA. (A) Aspiration cytology: Malignant mesenchymal cells, adjacent bone matrix, and spindle cells (Giemsa, obj. 40x). (B) Objective cytoinclusion 10x and (C) objective cytoinclusion 40x: High cellularity in cell clusters; malignant cells presenting preservation of the neoplastic architectural pattern; and morphological characteristics of neoplastic osteoblasts, adjacent bone matrix, and HE spindle cells. (D) Histopathological objective 10x and (E) Histopathological HE and 40x: High cellularity of malignant osteoblasts and preexisting bone trabeculae. There is a large proliferation of malignant spindle cells and production of nonmineralized bone matrix. (F) Cytoinclusion: Positive immunohistochemistry for osteopontin antibody (obj. 40x).

The cytological and tissue samples were evaluated by means of a statistical analysis of the data obtained using 2 × 2 contingency tables (Tables 3 to 5).

Table 3: Contingency table comparing cytological results via smears with histopathological findings from bone formations. This table shows the number of true positives, false positives, false negatives, and true negatives.

Histopathologic Smear	**+**	**-**	**Total**
+	11	0	11
-	0	0	0
Total	11	0	11

Table 4: Contingency table comparing cytological findings by cytoinclusion with histopathological findings from bone formations. This table shows the number of true positives, false positives, false negatives, and true negatives.

Histopathologic Cell Block	**+**	**-**	**Total**
+	11	0	11
-	0	0	0
Total	11	0	11

Table 5: Contingency table comparing cytological results by smear + cytoinclusion with histopathological findings from bone formations. This table shows the number of true positives, false positives, false negatives, and true negatives.

Histopathologic Smear + Cell Block	**+**	**-**	**Total**
+	11	0	11
-	0	0	0
Total	11	0	11

DISCUSSION

Eleven animals affected by OSA were selected for this study. The cytological and tissue specimens were processed by smearing and cytoinclusion, and the diagnosis

was confirmed using histological sections. According to Ryseff and Bohn (2012), cytological evaluation by aspiration puncture is a minimally invasive technique that has a precise diagnosis in a short interval of time. However, confirmation is made by histopathological examination.

Among the animals mentioned in Table 1, it can be observed that female dogs were more affected than male dogs. However, there was no statistical difference, and this result can be associated with our low number of samples. Neuwald et al. (2006) reported that male dogs have a higher occurrence of OSA than female dogs. According to Langenbach et al. (2001), the St. Bernard, Rottweiler, and Danish breeds are exceptions, with a higher incidence in female dogs. However, in other studies, no sexual predisposition has been observed in the frequency of this tumor (LANGENBACH et al., 2001).

The location of the lesion is an important indicator for assessing the prognosis (CAVALCANTI, 2007). The OSA location in our study is in accordance with Cavalcanti et al. (2004), who reported that the distribution of lesions according to frequency was greater in the radius and ulna (26.8%), followed by the femur (24.8%), tibia, scapula, humerus, and finally phalanges.

According to Nelson and Couto (2006), OSAs located on the scapula have a poor prognosis, and reports of increased survival occur when they affect the carpus and tarsal joints owing to amputation.

The majority of the animals had an age range of 8 to 13 years (nonsignificant result), with a mean age of 11 years. The literature cites that the disease affects elderly animals (average age of 8 years), but there are also reports indicating that the disease also affects young dogs (OLIVEIRA and SILVEIRA, 2008). St. Bernards, Irish Setters, Rottweilers (SILVEIRA, 2005), Dobermans (COOLEY et al., 2002), German Shepherds, Golden Retrievers, Boxers, Labradors, and Mastiffs are the most affected dog breeds (OLIVEIRA & SILVEIRA, 2008; ANGSTADT et al., 2011). In the present study, the disease was predominant in the Pit Bull and Rottweiler breeds, but it was also diagnosed in other breeds such as the SRD (nonbreed), Golden Retriever, German Shepherd, Akita, Siberian Husky, and English Setter. Bersano (2006) reported that dogs weighing more than 15 kg or greater than 36.5 kg are more likely to develop the tumor. Small dogs are rarely affected because of the early closure of growth plates (KRAJARNG et al., 2012). In the present study, the Pinscher breed was also affected.

Individual tumor cells vary in shape from round to fusiform and generally differ greatly in size. They often have classic cytological features of malignancy, such as in cardiomegaly and anisokaryosis, and large and multiple nucleoli that differ in size. It may also have clear vacuoles in the cytoplasm (FIELDER and MAHAFFEY, 2009). Most of the animals described in this study were affected by osteoblastic OSA.

The identification of osteoblast islands surrounded by tumor cells for osteoblastic OSA corroborates the findings of Fielder and Mahaffey (2009). For fibroblastic OSA, the presence of fusiform tumor cells with extensive areas of the osteoid matrix is a defining characteristic of the tumor classification of OSA defined by Meuten (2002).

In veterinary medicine, the delay in the use of cytology in clinical practice is mainly due to the ignorance of its diagnostic utility and the insecurity associated with its interpretation. The images that cytology provides (2D images) allow for adequate cellular detailing and the observation of cellular limits and their junctions (Ferreira, 2008). However, cytological specimens do not accurately reflect architectural parameters, and a cytological description of the architecture of the lesion is limited to isolated cellular information (MASSERDOTTI, 2006). Cellular arrangements are relevant in the evaluation of histological sections.

The arrangement of the cells in the cytoinclusion samples may mimic the arrangement thereof in the histological sections when harvested and prepared appropriately, with an alternative to performing the technique. Paraffin-embedded cytological samples usually preserve the architectural characteristics of the tissue within a cell population or even explicitly links the different neoplastic or nonneoplastic cellular populations, thus contributing to the diagnosis (ROFAGHA; SHECKET, 2002). Cytoinclusion is also indicated in the case of sample limitation, allows embedding, and has the advantage of providing a database, thereby maximizing information from the restricted material available (Kulkari et al., 2000). Moreover, cytoinclusion is faster, safer, and less expensive than a biopsy procedure.

According to Zanoni et al. (2013, 2012), few studies have been performed on cytoinclusion in the field veterinary medicine. Cytological samples were evaluated by smears and tissue samples by performing a statistical analysis of the data obtained using 2 × 2 contingency tables. These tables were also used for the evaluation of cytological samples via cytoinclusion and tissue culture.

We believe in the importance of the aspiration cytology exam as fundamental to the prognosis of the animal. This exam led to 100% diagnostic concordance in smears in the present study in relation to histopathology. When smear and cytoinclusion are considered in association, an "easiness" in the understanding of the etiological process, such as defining and subclassifying the diagnosis, was identified with a sensitivity of 100%. According to R.A Hegazy and A.A Hegazy (2013), cytoinclusion was introduced to improve the accuracy and interpretation of the diagnosis.

Vinayakamurthy et al. (2016) compared cytoinclusion with aspiration cytology in 66 human patients presenting with abdominal masses and obtained a sensitivity of 91.6% and specificity of 88.88%. The same authors reported that they were able to provide a definitive cytopathological diagnosis in all 66 cases and were able to increase the sensitivity to 100% when associated with cytological examinations with cytoinclusion samples. In the present study, we obtained 100% sensitivity and specificity.

The specificity calculation was compromised because of the absence of true negatives. However, the descriptive analysis of the cases confirmed that the specificity of the cases was 100% for both analyses.

Some studies affirm that the results obtained by the cytoinclusion method are more satisfactory than those obtained with the conventional cytology owing to the greater efficacy in reaching the acquisition of adequate material for establishing a cytopathological diagnosis concordant with the histological examination (NODA et al., 2010; ZANONI et al., 2013; PAIVA et al., 2014).

Owing to greater architectural preservation, the evaluation of the cytoinclusion samples is based on better established architectural relationships than on smear samples. This approach aids in the characterization of the process, as corroborated by the images of this study, which show a concordance of cytoinclusion with the histopathological examination wherein cell architecture preservation and tissue formation are present.

Several fixators have been used in the literature: buffered formalin (KASE et al., 2016), picric acid, Bouin's solution, Carnoy's fixative, B-5 mercury fixer, ethanol (ZITO et al., 1995; ZANONI et al., 2000), and agarose gel (ZANONI et al., 2010, 2013). This variety is directly related to the diversity of existing techniques, which have been adapted to the needs and specificity of each researcher (ZITO et al., 1995).

In this study, fixation with 1 mL of 95% alcohol was chosen and allowed to stand for five minutes for the fixation of the material in the syringe itself. Thereafter,

approximately 9 mL of 10% formaldehyde was aspirated and maintained for another 24 hours while keeping the syringe positioned upward for cell decantation. This technique is easier to perform; can be easily used in veterinary clinics, hospitals, and routine examinations; has a low cost; and requires less time for sample processing. In comparison with the agarose gel technique, this technique has a great advantage of convenience and low cost because it does not require the use of centrifugation, agarose gel, and Eppendorff devices, in addition to having no problems in making very dry samples.

According to Nathan et al. (2000), owing to the great variability of techniques tested and used to perform cytoinclusion, the comparison between them becomes impractical, thus generating significant difficulties in the standardization of a universal method for the processing and storage of materials for cytological examination. The same authors mentioned that the development and standardization of the cytoinclusion technique must meet the following requirements: maintenance of the tissue architecture and cellular morphology; optimization of the processing time of samples to avoid loss of quality of the material; sufficient reserve material for testing such as special staining and immunohistochemistry reactions; and development of a simple, low cost, and reproducible method.

In relation to immunohistochemical labeling, all samples were positive, thus demonstrating the efficacy of the use of OPN as an immunomarker in cytoinclusion samples. Osteopontin is an osteoblast-expressed flag that regulates bone matrix mineralization (ERIKSEN, 2010). Currently, molecular techniques can be performed directly on cytological smears (Schmitt and Barroca, 2012). In immunocytochemistry, several variables may affect the results of cytological preparations. Therefore, accurate and reliable results should be considered carefully. Special attention should be given to the type of material collected, type of fixative used, antibodies selected, and appropriateness of the controls. According to Fowler and Lachar (2008), internal positive controls are usually difficult to obtain in cytological samples. Immunohistochemistry in cytoinclusion samples shows superior results compared with cytological smears (VAYAYAKAMURTHY et al., 2016).

Many sections can be cut owing to the embedding of the material, thus reducing the number of artifacts and facilitating the making of other slides. The high cellularity allows for the evaluation of a large number of antigens, thus facilitating the confection of the technique. This is in line with the findings of Schmitt and Barroca (2012).

Vinayakamurthy et al. (2016) concluded that although there may be cases with cytoinclusion blotchiness, they may contain adequate diagnostic material, thus further enhancing diagnostic specificity and providing cellular architecture and additional sections for special staining and immunohistochemistry.

The use of biomarkers that specifically target OSA in dogs needs to be customized for the treatment of patients. Thus, we identified the potential genes that are active in the tumor by using a panel immunomarker to achieve better therapeutics as a function of this expression, provide better quality treatment, and increase the life expectancy of the animal (SELVARAJAH and KIRPENSTEIJN, 2010). Kase et al. (2016) reported that cytoinclusion is important in the use of immunocytochemistry.

CONCLUSION

The high sensitivity and specificity found for the immunohistochemical marking of osteopontin in OSA are associated with the morphological description between the cytomorphological diagnosis of cytoinclusion and histopathological diagnosis. This information affirms that the cytoinclusion technique is effective and a good alternative for preserving the cell architecture compared to cytological examination. Cytoinclusion enables safe analyses when researching the prognostic markers of bone tumors, requires less time, has a low cost, and can be used routinely in veterinary clinics and hospitals to obtain a material that can be archived. In this study, we conclude that the cytoinclusion method is effective for the diagnosis of OSA in dogs.

REFERENCES

Angstadt, A. Y.; Motsinger-Reif, A.; Thomas, R.; Kisseberth, W. C.; Guillermo Couto, C.; Duval, D. L.; Nielsen, D. M.; Modiano, J. F.; Breen, M. Characterization of canine osteosarcoma by array comparative genomic hybridization and RT-qPCR: signatures of genomic imbalance in canine osteosarcoma parallel the human counterpart. Genes, Chromosomes & Cancer, V. 50, no. 11, p. 859-874, 2011.

Berquó, E. S.; Souza, J. M. P.; Gotlieb, S. L. D. Biostatistics. 2. ed. São Paulo: E.P.U, 2001. 350 p.

Bersano, P. R. O. Immunohistochemical expression of cyclooxygenase-2 (Cox2) and quantification of organized nucleolar regions (NORs) in the different histological patterns of canine OSA, 2006. 120f. Dissertation

(Magister Scientiae) - Federal University of Viçosa.

Briffod, M.; Hacène, K.; Le Doussal, V. Immunohistochemistry on cell blocks from fine-needle cytopunctures of primary breast carcinomas and lymph node metastases. Modern Pathology, V. 13, no. 8, p. 841-850, 2000.

Cavalcanti, J. N.; Amstalden, E. M. I.; Guerra, J. L.; Magna, L. C. OSA in dogs: clinical-morphological study and prognostic correlation. Brazilian Journal of Veterinary Research & Animal Science. V, V. 41, no. 5, p. 299-305, 2004.

Canine, J. N. CAVALCANTI OSA: histopathological,

immunohistochemical and proliferative activity study, 2007. 96f. Thesis (Doctoral Degree in Medical Sciences) - State University of Campinas, UNICAMP.

Cooley, D. M.; Beranek, B. C.; Schlittler, D. L. et al Endogenous gonadal hormone exposure and bone sarcoma risk. Cancer Epidemiology, Biomarkers & Prevention, V. 11, no. 11, p. 1434-1440, 2002.

Daleck, C. R. et al Retrospective study of primary osteosarcoma of pelvic bones in dogs over a 14-month period. Brazilian Journal of Veterinary Research & Animal Science, V. 43, no. 1, p. 125-131, 2006.

Eriksen, E. F. Cellular mechanisms of bone remodeling. Reviews in Endocrine & Metabolic Disorders, V. 11, no. 4, p. 219-227, 2010.

Ferreira, L. F. G. R. B. Comparative study between cytological and histopathological analyzes in the diagnosis of canine tumors, 2008 100f. Master's Dissertation Integrated in Veterinary Medicine- Faculty of Veterinary Medicine, Technical University of Lisbon: Lisbon.

Fielder, S. E.; Mahaffey, E. A. Skeletal muscle system. In: Cowell, R.L.; Tyler, R.D.; Meinkoth, J.H.; DeNicola, D.B., editors Cytological Diagnosis and Hematology of Dogs and Cats. 3rd ed., New York, USA, 2009. P. 210.

Fowler, L. J.; Lachar, W. A. Application of immunohistochemistry to cytology. Archives of Pathology & Laboratory Medicine, V. 132, no. 3, p. 373-383, 2008.

Handa, U. et al Fine needle aspiration cytology in the diagnosis of bone lesions. Cytopathology, V. 16, no. 2, p. 59-64, 2005.

Hegazy, R. A.; Hegazy, A. A. FNAC and Cell Block study of thyroid lesions. Universal Journal of Medical Science, V. 1, no. 1, p. 1-8, 2013.

Heyman, S. J.; Diefenderfer, D. L.; Goldschmidt, M. H. et al Canine axial skeletal osteosarcoma a retrospective

study of 116 cases (1986-1989). Veterinary Surgery, V. 21, no. 4, p. 304-310, 1992.

Kase, S.; Namba, K.; Iwata, D.; Mizuuchi, K.; Kitaichi, N.; Tagawa, Y.; Okada-Kanno, H.; Matsuno, Y.; Ishida, S. Diagnostic efficacy of cell block method for vitreoretinal lymphoma. Diagnostic Pathology, V. 11, p. 29, 2016.

Krajarng, A.; Nilwarankoon, S.; Suksamrarn, S.; Watanapokasin, R. Antiproliferative effect of a-mangosteen on canine osteosarcoma cells. Research in Veterinary Science, V. 93, no. 2, p. 788-794, 2012.

Langenbach, A. et al Extraskeletal osteosarcomas in dogs: a retrospective study of 169 cases (1986-1996). Journal of the American Animal Hospital Association, V. 34, no. 2, p. 113-120, 1998.

Loukopoulos, P.; O'Brien, T.; Ghoddusi, M.; Mungall, B. A.; Robinson, W. F. Characterization of novel canine osteosarcoma cell lines producing high levels of matrix metalloproteinases. Research in Veterinary Science, V. 77, no. 2, p. 131-141, 2004.

Masserdotti, C. Architectural patterns in cytology: correlation with histology. Veterinary Clinical Pathology, V. 35, no. 4, p. 388-396, 2006.

Maxie, M. G. Pathology of Domestic Animals. 1. 5th ed, Sauders-Elesevier: Oxford, 2007. Pp. 110-177.

Meuten, D. J. Tumors in Domestic Animals. 4.d., Iowa State Press: Iowa, 2002, 778p.

Mueller, F.; Fuchs, B.; Kaser-Hotz, B. Comparative biology of human and canine osteosarcoma. Anticancer Research, V. 27, no. 1A, p. 155-164, 2007.