Processing and characterization of canine mixed mammary tumor using transmission electron microscopy

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Abstract
Canine mammary gland tumors represent the second most frequent type of neoplasm in dogs, being an important problem within veterinary medical field. Canine mixed mammary tumors are the most common; the use of a transmission electron microscope (TEM) can contribute as a tool in its diagnosis by determining the characteristics of cellular components from numerous neoplasms. The aim of this study was to characterize cytologically canine mammary mixed tumor by the use of the TEM. A biopsy collected from an 11 years old bitch Shih-Tzu and analyzed by histopathology was used for ultrastructural analysis. Specimens obtained were double stained using uranyl acetate and lead citrate prior to observation in the TEM. The protocol established to transmission electron microscopy observation allowed the identification of main cellular characteristics of canine mixed mammary tumors; however, it was not possible a detailed visualization of the organelles due to the preservation of the biopsy in formaldehyde.

KEYWORDS
canine mixed mammary tumors, histopathology, transmission electron microscopy

1 INTRODUCTION

The mammary glands are modified sweat glands whose fundamental function is the nutrition of neonates during lactation. In canines, it can be found from 10 to 14 mammary glands, distributed in two rows. Structurally they are composed of lobes, which have lobules and internal ducts, upholstered by epithelial and myoepithelial cells (González & Ugalde, 2012).

Mammary tumors in dogs represent the most frequent neoplasm, presenting almost exclusively in females; however, sterilization in females decreases significantly the risk of having breast tumors specially an early ovary-hysterectomy (Cassali et al., 2012). In addition, age is also a factor related to the development of tumor, the majority of neoplasms are developed in canines from 7 to 13 years old (Cassali et al., 2012).

Mixed mammary tumors in dogs are characterized by cells composed of epithelial, that is, luminal and/or myoepithelial, and mesenchymal components that develop cartilage and bone, although sometimes there is fibrous tissue (Aburto et al., 1997).

The use of transmission electron microscopy allows studying biological samples at the cellular and subcellular level, representing a fundamental tool in the diagnosis of pathologies. However, its success depends on the thickness of the observed sample. To visualize biological samples in a transmission electron microscope (TEM), it is essential to follow specific protocols, which provide details of materials used and conditions to keep intact cellular and subcellular structures.

The aim of this study was to adapt a protocol and characterize cytologically canine mammary mixed tumor using transmission electron microscopy.

2 MATERIALS AND METHODS

The biopsy used in this study was taken from an 11 years old bitch Shih-Tzu, obtained in the Veterinary Anatomopathological service laboratory in Quito (Histo-Dx-Vet) and conserved in formaldehyde 10%. The protocol used was modified from the one developed by Kuo (2007), cutting the extract tissue into pieces of about 2 mm of length. Fixation was performed suspending the samples in glutaraldehyde at 4% with 0.1 M sorenson phosphate buffer pH 7.2 for 2 hr. Subsequent three washes were performed using 0.1 M sorenson phosphate buffer 7.2 pH at 4°C for 15 min each wash. Post-fixation was
carried out in osmium tetroxide 1% with 0.1 M sorenson phosphate buffer 7.2 pH for 1 hr at 4°C. Subsequently, samples were rinsed three times with distilled water for 2 min each, followed by dehydration in series of ethanol at 30, 50, 60, 70, 80, 90, and 99% at 4°C for 30 min each, and then placed in ethanol at 99% overnight at 4°C. Finally, intermediate solvent was used placing the sample in propylene oxide at 4°C twice for 5 min each.

Infiltration and polymerization were done according to Burghardt and Droleskey (2006), using an epoxy resin composed of 20 ml Epon 812, 16 ml dodecenylsuccinic anhydride, 8 ml of methyl nadic anhydride, and 1.3 ml benzydimethylamine. Infiltration samples were placed in a mixture of 50% epoxy and 50% propylene oxide at room temperature and shaking them for 1 hr, and then the resin was replaced for 75% epoxy and 25% propylene oxide at room temperature and shaking for 1 hr. Then, resin was removed and replaced with 100% epoxy using the same parameters described above. The polymerization was performed by placing the samples in freshly prepared 100% epoxy and incubated at 60°C for 2 days.

Ultrafine sections were obtained by the use of an ultramicrotome with 1 mm/s speed and 100 nm thickness. Finally, staining started with the use of uranyl acetate at 2% with pH 4, several drops of the reagent were deposited on a piece of paraffin in a petri dish, and grids were placed with the sample face down at room temperature for 5 min protected from light. Then, four washes were carried out in boiled distilled water, and the mesh was immersed from 6 to 8 times in each wash. Subsequently, grids were placed face down on drops of lead citrate contained in a petri dish and surrounded by sodium hydroxide pellets, to absorb the carbon dioxide at room temperature for 3 min, the grid was removed and the same washing described above was applied. Pictures were taken using a FEI Tecnai G2 Spirit TWIN, and a Tescan Mira 3 FEG-SEM transmission mode (STEM).

3 | RESULTS

The patient was diagnosed with mixed mammary tumor of benign nature. The histopathology analysis performed on the biopsy showed the presence of connective tissue with proliferation of myoepithelial cells, several layers of epithelial cells, lining the irregular tubules of the lobes, and metaplasia toward hyaline cartilage and bone (Figure 1).

Instrumental errors were evidenced when the sample was sectioned, that is, lack of sharpness of the glass blades and irregularities in the edge of the diamond blade. This produces scratches in the sectioned tissue, the depth of each rayon was related to the speed of sectioning. An illustrative example is shown in Figure 2a,b, where the speed of sectioning was varied from 0.5 to 1 mm/s, respectively.

Figure 3a–f shows the various cellular characteristics identified in mixed tumors, which demonstrate a variety of tissues including epithelial cells, connective fibrous tissue, cartilage, bone, and neoplastic proliferation of myoepithelial cells.

4 | DISCUSSION

The histopathology analysis did not allow observing specific cytology in detail. The TEM can be used for confirmatory diagnosis, although the
main disadvantages are the cost, availability of the equipment, and time consuming.

The biopsy was preserved in formaldehyde at 10% for 10 days until its analysis by TEM. Previous studies mentioned that in spite of the tissue preservation capacity of formaldehyde, in many cases it has been responsible for a significant loss of structural details of the sample (Stirling, Curry, & Eyden, 2013). In our study, the images obtained by TEM allowed identifying several structural characteristics of the tumor, mainly cellular and nuclear, although characteristics of organelles were difficult to determine; this could be related to the preservation of the tissue in formaldehyde or due to the chemicals used during the procedure. The methanol content in the formaldehyde can denature proteins and is also considered as a coagulative fixative (Ghadially, 1980).

Technical problems presented during the experiment could also be involved in several samples that had to be observed under scanning electron microscope with TEM function, also known as STEM.

The mammary gland neoplasms are characterized by excessive undefined and disordered cell proliferation, determining the formation of masses that present a varied size and shape known clinically as tumors; mixed canine mammary gland tumors exhibit histological characteristics common with salivary gland tumors in humans, that is, pleomorphic adenomas, which are derived from exocrine glands (Ballance, El-Naggar, Grignon, Ayala, & Romsdahl, 1990).

In our study it was found various cellular characteristics identified in mixed tumors, including epithelial cells, connective fibrous tissue, cartilage, bone, and neoplastic proliferation of myoepithelial cells. According to Aburto et al. (1997), mixed tumors also present myxoid tissue as a tissue component, which may serve as a precursor for cartilage (Egerbacher & Böck, 1997).

The structure of the mammary gland is lined internally by luminal epithelial cells, and externally by a layer of myoepithelial cell; luminous epithelial cells are characterized by their secretory properties, lack intercellular space, and present cuboidal or columnar shape with scarce cytoplasm, elongated or oval central nucleus, with marginal heterochromatin and round nucleolus in contact with the nuclear envelope (Jatoi & Kaufmann, 2010), as shown in Figure 3a.

The glandular epithelium of mammary glands is characterized to be exocrine, because its cells have the ability to release substances through a system of ducts, among the main inclusions of epithelial origin are granules of mucigen as shown in Figure 3b, which present a single limiting membrane with variation in its internal content; being able to vary in density and substructure, depending on their differences with respect to their molecular composition or to the binding protocol (Eyden, Sankar, & Liberski, 2013).

Myoepithelial cells or myoepithelium are cells of epithelial nature, whose function is to release milk stored in the alveoli and expelled through the ducts galactophores (Akiyoshi, Uchida, & Tateyama, 2004), Figure 3c shows a cellular morphology characteristic of myoepithelial differentiation, as it exhibits irregular and dilated form, ovoid and irregular nucleus, apparent nucleolus, and presence of heterochromatin; characteristics described by Aburto et al. (1997) in previous studies. Myoepithelial cells may have a spindle or star shape, which proliferate in interstitial areas of mixed tumors, as shown in Figure 3d; which have abundant mucinous stroma, and in some cases are accompanied by chondroid changes (Akiyoshi et al., 2004).

Myxoid tissue is known in pathological diagnosis as a constituent of benign tumors, characterized to be avascular, it presents cells in star or elongated form with thin cytoplasmic extensions, oval nuclei, intermediate cytoplasmic filaments arranged in bundles that occupy a large amount of the cytoplasm, a small quantity of organelles, and collagen and elastic fibers in their extracellular matrix (Egerbacher & Böck, 1997). In Figure 2a,b, it can be observed a myxoid cell present in benign mixed tumor of our patient, which shows the characteristics previously described.

The cartilaginous tissue of benign mixed tumors is characterized by low or moderate amount of chondroblasts and chondrocytes, which rarely exhibit morphological alterations, hyaline cartilaginous tissue being...
the main constituent of this type of tumors (Cassali et al., 2012). Chondroblasts are metabolically active cells, which enter by mitotic divisions, originate daughter cells responsible for the synthesis of the extracellular matrix, being surrounded by a cartilaginous cavity known as a lagoon or chondroplast, and take the name of chondrocytes, those ones can be divided into the lagoon forming contiguous sets called isogenic groups.

FIGURE 3 TEM and STEM images from the biopsy of an 11 years old bitch Shih-Tzu using ultrathin cut 100 nm. (a) Luminous epithelial cells, cut at 1 mm/s. (b) Secretion granules in epithelial cells, cut at 1 mm/s. (c) Myoepithelial cell. (d) Transverse section of chondrocytes located within their respective lagoons distinguishing large lipid inclusions, cut at 1 mm/s. (e) Spindle-shaped chondrocyte located in tumor periphery, cut at 0.5 mm/s. F. Fibroblast with cellular processes, cut at 1 mm/s. Images (a–d) were observed under STEM, images (e,f) were observed with TEM. TEM = transmission electron microscope, STEM = scanning electron microscope with TEM function. [Color figure can be viewed at wileyonlinelibrary.com.]

CELLS RESPONSIBLE FOR SYNTHESIZING THE PRECURSORS OF COLLAGEN AND ELASTIC FIBERS ARE KNOWN AS FIBROBLASTS, WHICH IN THEIR ACTIVE STATE SHOW A STAR-SHAPED FORM WHEN SHOWING HIGH SYNTHETIC ACTIVITY, OR FUSIFORM FORM (FIGURE 3F); WHEN THESE CELLS ARE LOCATED BETWEEN COLLAGEN FIBERS, THEY CAN EXHIBIT AN EXTENSIVE OR ELLIPTICAL NUCLEUS WITH CHROMATIN AGGLOMERATIONS, ONE OR TWO NUCLEOLI AND ABUNDANT ROUGH ENDOPLASMATIC RETICULUM IN THEIR CYTOPLASM (BONUCCI & MOTTA, 1990). ACCORDING TO GHADIALLY (1980), SOME OF THE FIBROBLASTS PRESENT IN BENIGN NEOPLASMS HAVE A SPINDLE-SHAPED CELL WITH SEVERAL CELLULAR PROCESSES; IN FIGURE 3F APPRECIATED SMALL AMOUNT OF CYTOPLASM AND A HYPTROPHIC NUCLEUS CAN BE SEEN.


IN CONCLUSION, THE PROTOCOL ESTABLISHED PRIOR TO MICROSCOPIC OBSERVATION ALLOWS A GREAT EXTENT THE IDENTIFICATION OF THE MAIN STRUCTURES OF THE CELLS ANALYZED, BEING THESE CELLULAR AND NUCLEAR MORPHOLOGY; HOWEVER, IT WAS NOT POSSIBLE TO OBTAIN A DETAILED VISUALIZATION OF THEIR ORGANELLES, PROBABLY DUE TO THE PRESERVATION OF THE SAMPLE IN FORMALDEHYDE.

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CONFLICT OF INTEREST

THE AUTHORS DECLARE THAT THEY HAVE NO CONFLICT OF INTEREST.

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