Characterization of canine mammary tissues combining XRD and FTIR techniques

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Abstract

Dogs are the most abundant companion animals worldwide. Breast cancer is the most recurrent disease that affects those animals, due to the recent increase in care spared to food and health. Studies indicate that neoplastic tissues undergo structural and molecular changes that can be identified by analytical techniques. X-ray diffraction (XRD) identifies the molecular structural components of a sample. In addition, Fourier-transform Infrared Spectrophotometry (FTIR) identifies the vibration of molecules in a sample through infrared absorption. These two techniques complement each other, allowing the identification of molecular and supramolecular components in a given sample. XRD and FTIR were used to investigate the changes in tissue structure in different length scale associated with breast cancer in canine mammary tissues. A total of 83 samples were evaluated, being 58 neoplastic (malignant and non-malignant) and 25 normal or non-neoplastic, according to the histological classification by a veterinarian pathologist. X-ray scattering profiles were determined using the commercial diffractometer Shimadzu XRD-7000 with a copper anode at the momentum transfer range of $4.27 < q\ (nm^{-1}) < 50.18$. FTIR spectral profiles were acquired using a Varian spectrometer 640-IR with a Pike Miracle ATR (attenuated total reflection) and a ZnSe crystal. Absorption bands corresponding to lipids and fatty acids (adipose tissue) between 1750 and 3050 cm^{-1}, collagen fibers (1180-1300 cm^{-1}) and amides I and II (1500-1650 cm^{-1}), which compose the fibroglandular tissue, were determined from the FTIR data. These results corroborate the findings in XRD, in which the peaks found (13.9 nm^{-1} and 20.1 nm^{-1}) correspond to the fatty acids and water present in adipose and fibroglandular tissues, respectively. Therefore, the complementarity between XRD and FTIR is crucial to identify and characterize the morphological changes in canine mammary tissues structures related to the tumor growth in different length scale.

Keywords: Canine breast cancer; X-ray diffraction (XRD); Fourier-transform Infrared Spectrophotometry (FTIR).

1. Introduction

Canine breast cancer is the most recurrent type of cancer affecting female dogs. This cancer is caused by different factors, like hormone dependence, feeding quality and age, the last one being a result of greater care by tutors. In the last years, the increased care towards domestic animals, including better quality food, lifestyle and frequent visits to the veterinarian largely increased the canine life expectation [1].

The neoplasm is an abnormal growth of cells that do not cease with the initial stimulus. Some cellular alterations are not considered neoplasms, such as hyperplasia, hypertrophy, atrophy, and metaplasia. These are changes that regress their homeostasis state when the stimuli is removed, which may be hormonal, physical, chemical, and so forth [2].

Neoplasms are classified as either benign or malignant. Benign neoplasms are more controlled, with delimitation of size and slow growth in comparison to those malignant, which are generally more aggressive in growth and metastasis [3].

Tumors cause structural and chemical changes in tissues as they develop. Canine breasts are constituted by adipose and fibroglandular tissue. Adipose tissue is mainly composed of fatty acids and triglycerides and is responsible for mechanical protection. Collagen fibers and the mammary glands are the principal components of the fibroglandular tissue [4].

Female dogs usually have five pairs of breasts, forming two mammary chains. Because of this, it is not difficult to find more than one histological type of neoplasm in the same mammary chain, as well as hyperplasias and meta-

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plasias, considered pre-neoplastic elements [5].

Clinically, the detection of these pathologies is performed by histological assessment of tissue specimens after biopsy surgery, by a veterinarian pathologist. The histopathological analysis determines the type of neoplasm, and therefore the prognosis and best treatment for the canines. Nevertheless, the efficacy of the treatment is limited by the lack of information about the morphological alteration of the mammary tissues due to the type and stage of the tumor [6]. Furthermore, due to the physiological similarities between canine and human mammary tissues, the study of canine tumors as a model for humans is essential for translational medicine [6].

X-ray Diffraction (XRD) and Fourier-transform Infrared Spectrophotometry (FTIR) have been widely used to identify the structural elements of several types of samples, including biological tissue [7, 8]. While XRD allows identifying the molecular structural components of a sample [9, 10], FTIR is sensitive to the conformation and change in expression of various biomolecules [11].

Therefore, this study aimed to identify the morphological changes in canine mammary tumors in different length scales by combining XRD and FTIR.

2. Materials and methods

2.1. Canine breast tissue samples

A total of 83 samples were evaluated, being 58 neoplastic and 25 normal, according to the histological report. They were provided by the Laboratory of Veterinary Pathology of the Federal University of Paraná and belong to a sample bank. Their use was approved by the Committee on Ethics in the Use of Animals of the Federal University of Technology - Paraná (UTFPR), protocol number 2016-009. The samples were kept in a 10% formaldehyde solution at room temperature. For the analysis, they were drained for 10 seconds in order to reduce their liquid content. The canine mammary tissue fragments were cut into a circular shape with 21.0 mm diameter and 4.0 mm height, which perfectly fits in the sample-holder used.

2.2. XRD experimental arrangement

A commercial diffractometer Shimadzu XRD-7000, represented in Figure 1), with a Copper anode ($Z_{Cu} = 29$, $K\alpha_{Cu} = 8.04$ keV) was used. Scattering profiles were acquired between $6^o < 2\theta < 76^o$ with steps of $1/3^o$ and 20 s of exposure per step, resulting in a momentum transfer interval of $4.27\ nm^{-1} < q\ (= 4\pi \sin(\theta/2)/\lambda) < 50.18\ nm^{-1}$. The equipment had a system of slits that kept the irradiated area constant (3.5 mm × 19 mm) and a graphite monochromator to select elastic scattered photons. A specially designed sample-holder was used, made of lactic polyacid plastic, with the internal dimensions as cited for the samples. During the analysis, the samples were covered by a PVC film in order to avoid humidity loss and to provide a uniform surface. The obtained intensities (scattering profiles) were corrected for spurious contribution, self-attenuation, polarization, irradiated volume, and detector efficiency, as described in [9].

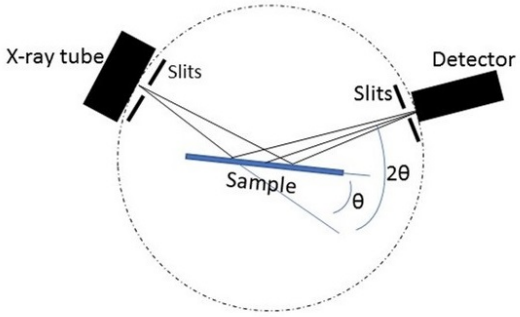

Figure 1: XRD experimental arrangement with a Copper X-ray tube, sample, NaI detector and divergent slits.

2.3. FT-IR experimental arrangement

The infrared spectrometer with Fourier Transformation Varian spectrometer 640-IR coupled to a Pike Miracle ATR (attenuated total reflection), and a ZnSe Crystal was used (Figure 2). It produces two mid-infrared radiation beams, one fixed and the other variable, which allows faster and high resolution acquisition (multiple scan). The samples were positioned on the crystal center and clamped firmly in order to improve the contact between the sample and the crystal. Formaldehyde solution was subtracted for signal reduction, thereby improving the identification of sample absorption bands. Using an incidence angle of 45° the penetration depth in the sample was around 2.0 μm. 32 repetitions for each sample with a resolution of 4.0 cm^{-1}, resulted in an reading range of 4000 cm^{-1} to 650 cm^{-1}, expressed in terms of transmittance.

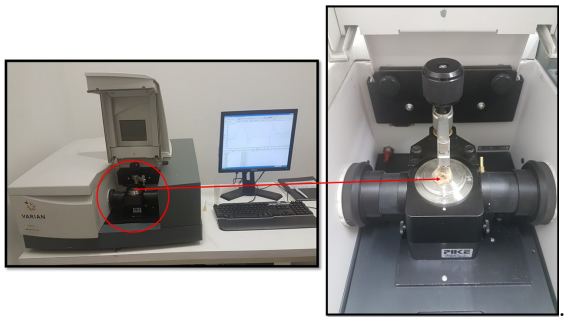

Figure 2: FTIR experimental arrangement, highlighting the ATR accessory with sample positioned.

3. Results and discussion

In the X-ray elastic scattering profiles, presented in Figure 3, two main peaks are clearly observed, at q = 13.9 nm^{-1} and q = 19.8 nm^{-1}. The first one corresponds to an

electron density correlation of 4.5Å, thereby related to the content of fatty acids (adipose tissue). Adipose tissue is present basically in all canine breasts, mainly the ones in the thoracic and inguinal portions, responsible for mechanical protection to the breast. Electron density correlation of 3.0Å is obtained from the peak at q = 19.8 nm^{-1}, which is associated with the water content, the main component of glandular tissue. Due to the complementarity of functions, the term fibroglandular tissue is often used to represent glandular and fibrous tissue. Therefore, XRD allows to drawn a relation between the adipose and fibroglandular content in normal and neoplastic canine mammary tissue.

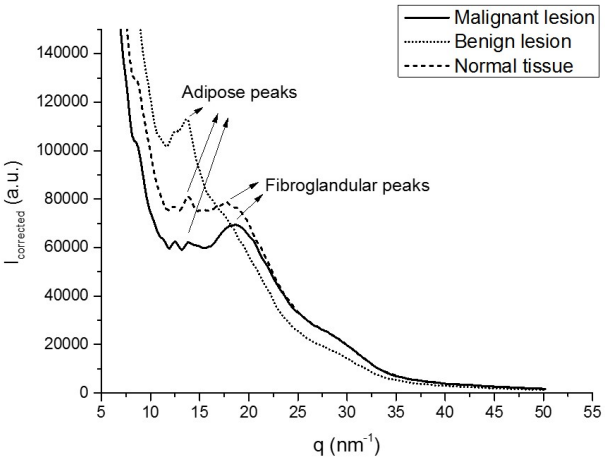

Figure 3: Characteristic elastic scattering profiles for canine mammary tissues, including normal, benign and malignant samples.

The results for FTIR are presented at Figure 4. The area between 1100 cm^{-1} and 3000 cm^{-1} are typical bands of absorption (known as fingerprints) to canine mammary tissue (benign, malignant and normal adipose).

Malignant tissue presented bands of absorption in the region of the collagen molecules (1165 cm^{-1}, 1237 cm^{-1} and 1283 cm^{-1}). The peaks found at 1457 cm^{-1}, 1542 cm^{-1} and 1636 cm^{-1} are characteristic from protein bands like amides II and amides I. Proteins play a fundamental role for live organisms. They are composed of many amino acids, forming a linear polymer. Proteins can have a tridimensional configuration caused by folds, which forms secondary structures that can be identified by FTIR [12]. Amides are polypeptides and their identification, in most cases, can be drawn from the C=O bound to amide I and the N-H bound to amide II. Amides I and II can provide important structural information about the analyzed sample, especially amide I due to its higher sensibility and because it brings information about the protein folds and hydrogen bounds, revealing the presence of disorders at molecular level [13]. It is known that neoplasias cause structural changes in tissue, so the identification of these changes can function as a biomarker to canine mammary neoplasias. There is also an important peak at 1742 cm^{-1}, which corresponds to lipids. Absorption bands from fatty acids, related to CH2 and CH3 bounds can be spotted at 2850 cm^{-1} e 2911 cm^{-1}, respectively [14].

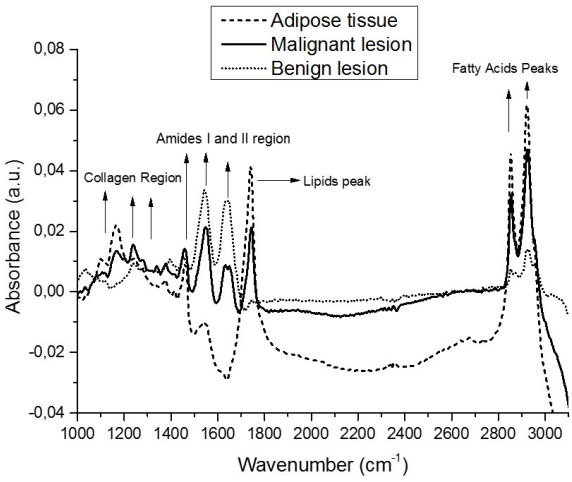

Figure 4: Absorption bands identified with ATR-FTIR for normal, benign and malignant canine breast tissues.

When analyzing the results for benign tissues, there was only one peak correspondent to the absorption band of collagen, at 1235 cm^{-1}. However, the region correspondent to amides I and II showed higher absorbance in comparison to malignant tissue. The higher intensity from protein peaks could meàn that they are folded and arranged in a different way than other tissues.

Amides I and II absorption bands are missing in the normal adipose tissue profiles, proving this tissue is mainly constituted by lipids and fatty acids.

Small differences in the absorption band's values for different types of tissues can be linked to the way proteins and other compounds in the sample are arranged. Also, the presence or absence of certain peaks might indicate the presence of compounds generated by molecular changes. Therefore, the analysis of these peaks are an effective way to characterize tissues [14].

Figure 4 shows the FTIR spectra from benign, malignant and normal canine mammary tissues. Although the absorption band's values can slightly vary because of their spatial arrangement, as well as their forms of bond, the typical vibration modes allow the identification of the molecules [15]. The band between 1500 cm^{-1} and 1650 cm^{-1} corresponds to the content of amide I and II, proteins that constitute the fibers present in fibroglandular tissues. Likewise, the absorption bands related to collagen fibers are observed between 1180 cm^{-1} and 1300 cm^{-1}. The adipose content is identified by the bands at 1750 cm^{-1} and between 2800 cm^{-1} and 3050 cm^{-1}, corresponding to the vibration of fatty acids and lipids, respectively [4, 16]. DNA's absorption bands were also identified, around 1230 cm^{-1}, highlighting the potential of FTIR in identifying supramolecular structures and their possible changes, e.g.,

due to breast tumor growth [17].

4. Conclusion

Here we presented the complementarity of XRD and FTIR in characterizing normal and neoplastic canine mammary tissues in different length scale. XRD allowed the identification of the small molecules, fatty acids and water, which compose adipose and fibroglandular tissue, respectively. Looking at submolecular bonds and functional groups, FTIR was able to characterize larger molecules such as lipids as well as amide II and III and collagen of the same mammary tissues. Therefore, the data provided by the combination between XRD and FTIR have great potential for improving the knowledge of canine mammary tumor growth, as well as for the development of new therapeutic methods based on the molecular and supramolecular changes.

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