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Histochemical study of fibrillar proteins of the extracellular matrix in benign and malignant mammary neoplasms in dogs

Estudo histoquímico de proteínas fibrilares da matriz extracelular em neoplasias mamárias benignas e malignas na espécie canina

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SUMMARY

The aim of the present study was to study some of the proteins that form the extracellular matrix of 54 benign and malignant mammary neoplasms in dogs, using histochemical methods: Picrosirius and polarization microscopy for collagenous fibers, Gordon -Sweats's method for reticular fibers and Weigert's fucsine-resorcine method for elastic fibers. A large variability in quantity, distribution and characteristics of the matrix components was observed in the different types of neoplasms. Collagen type I, III and elements of the elastic system had different distribution in benign and malignant neoplasms. The simple Picrossirius method and under polarization enabled visualization of collagen as thick fibers irregularly distributed in the stroma of carcinomas and in a more orderly and regular fashion in benign neoplasms. A smaller amount of thin fibers was observed in an irregular and random disposition in carcinomas and in a regular disposition in benign neoplasms . Under polarization, the fibers present different lengths, were yellowish or reddish and strongly birefringent, what suggested that they were collagen type I and in the middle of these fibers, other ones, pale, greenish and weakly birefringent, some of them thinner, possibly collagen type III were observed. In the stroma of carcinoma, fibers were mostly thick, strongly birefringent, yellowish or reddish, disposed in an irregular and random fashion, mainly in the central areas. In condrometaplasic areas, both in malignant and benign neoplasms, there was a collagen population composed by thin fibers in a parallel disposition, limiting narrow regions where condrocytes were aligned. Around this area, there was a collagen population formed by bundles of thick anastomosed fibers, irregularly disposed in carcinomas and orderly, in a parallel fashion in benign neoplasms. Under polarization demonstrated that this population, among condrocytes, was formed by weakly birefringent fibers, pale and yellowish, what suggested a collagen type II pattern. The use of reticular fibers staining by Gordon & Sweats, enabled a visualization of collagen as thin fibers disposed not only in the dense stromas but also in the loose ones. These fibers presented variable density, but were found mainly around acini and tubules. In relation to the presence elements of the elastic system in benign and malignant tumors, it may be observed that they predominate in the malignant ones, mainly in the pseudocapsule and around acini and tubules. Elements of the elastic system were not observed in the specimens when they were submitted to Weigert's staining without oxidation. When the same material was submitted to Weigert's staining with oxidation , oxytalan fibers were more evidently around acini and tubules, as well as in the pseudocapsule. Elements of the elastic system were in the ECM, both in samples submitted to staining with oxidation and without it and this was similar for benign and malignant tumors. Results of this study emphasize the profound structural changes in collagenous and fibrous components of the extracellular matrix elastic system of mammary neoplasms in dogs.

KEY-WORDS: Mammary neoplasms. Collagen. Dogs. Extracellular matrix.

INTRODUCTION

An ever growing importance is being attributed to the extracellular matrix (ECM) not only in normal processes, but also in neoplasms.

The clinical and pathological expression of tumors is a result of the combination of several factors related to the whole population of neoplasic cells, such as proliferation, invasion and the expression of cell markers, besides other factors directly expressed by the host, such as the inflammatory response, macromolecules in extracellular matrix and neovascularization[7;1].

Cells of higher multicellular organisms were embedded in an amorphous viscoelastic substance, a highly organized matrix made up of proteins and glycoconjugates. The most important component of this extracellular matrix is collagen, but elastin, proteoglycans, glycosamines, glycoproteins and several other proteins responsible for proliferation, differentiation, adhesion, migration and cell apoptosis were also significantly present[8,15].

More than 20 types of collagen have been described Martin and Timpl[17] and Olsen and Ninomiya[21]. They were

chemically, genetically and immunologically different. Differences were found in the primary composition of the molecules, in the type of extracellular aggregation and in the capacity of producing - or not - fibrilar structures[21].

Collagen type I, II, III, V and XI take part in the production of fibrils, molecules which were ordered in pattern called ¼ . They were encoded by homologous genes which present multiple exons (more than 50)[21] .

Supramolecular aggregates of triple helix fibrilar collagen usually contain more than one type of collagen and each heterotypical fibril is arranged in a different tissue pattern: parallel bands in the tendon; crossed layers in the cornea and spiral arrangement in lamellar bone[21].

Fibrilar collagen may interact directly with receptors in the cell surface or indirectly via other components of the extracellular matrix. Each type of inter-relationship of fibrilar collagen influences cell behavior and differentiation during embryonic development[21].

Collagen type I is predominantly found in the skin, bones, tendons, dentine, capsule of different organs, pericondrium and fibrous cartilage. The function of this type of collagen is to counteract tension. It is made up of two a1 chain (I) and one a2 chain (I). It is produced by fibroblasts, osteoblasts, odontoblasts and condroblasts. It forms fibrils that present average diameter equal to 78nm Montes , Junqueira [19], with a characteristic evident striated pattern. These fibrils were frequently found parallel to each other, forming fibers of 2 to 10 mm, the collagen fibers. Eventually, these fibers may group in an orderly fashion, forming a bundle of collagenous fibers.

Collagen type II is mainly found in hyaline and elastic cartilage, corneal stroma and vitreous humor. The molecule is constituted by three a1 (II) chains, highly associated to the proteoglycan in the extracellular matrix, what provides it with great resistance to the pressure to which cartilage were submitted[13]. This type of collagen is mainly produced by condroblasts, and fibrils present average diameter equal to 20nm[19].

Collagen type III is generally found in tissues or organs that also contain type I and which require a flexible structure, such as the liver, lungs, skin, spleen, muscles, uterus, blood vessels, intestine, etc. It is an homotrimerous of a1 (III) chains. Fibrils present average diameter ranging from 0.5 to 1.5 mm , and correspond to the reticular fibers of the conjunctive tissue[2] .This kind of collagen is produced by smooth muscle cells, fibroblasts, reticular cells, Schwann cells and hepatocytes[19].

Fibers from the elastic system were made up of elastic fibers, elauninic fibers and oxytalam fibers. Ultrastructurally, elastic fibers in mammals were constituted of a solid central cylinder made up of abundant amorphous and homogeneous material - elastin - involved by microfibrils from 10 to 12 nm diameters, which present an electrondense tubular profile, when transversely cut[23] . During their development, a microfibril bundle appears first, followed by amorphous material which is gradually deposited between microfibrils until total maturation of elastic fibers is completed[5]. It has been suggested that the three different types of fibers in the elastic system were a continuous series, which is chronologically initiated, during histogenesis, by oxytalan fibers, followed by the elauninic ones and ended by the mature elastic fibers.

The inter-relationships between the cells of the stroma with the ECM elements present deep implications in the biology of neoplasms, and may partially explain the latency periods in carcinogenesis and the establishment of metastasis. In a similar way, organic selectivity for the establishment of metastases, in several kinds of tumors, may be related to certain cell types from the stroma[4].

Interactions between neoplasic cells and their environment present, therefore, a new paradigm with important implications in cancer physiology and therapy.

MATERIAL AND METHOD

Blocks and slides with benign and malignant tumors of dogs were used in this trial. A total of 54 cases from the records in the Animal Pathology Sector at the Pathology Department in Faculdade de Medicina Veterinária e Zootecnia / USP, from 1936 to 1999 were analyzed. Of the 54 tumours cases, 3 were tubular adenocarcinomas simple,3 were tubular adenocarcinomas complex,3 were papillary adenocarcinomas simple, 3 papillary adenocarcinomas complex, 3 papillary cystadenocarcinomas simple , 3 papillary cystadenocarcinomas complex, 3 Solid adenocarcinomas simple, 3 solid adenocarcinomas complex, 3 spindle cells ,3 mucinous carcinoma ,3 anaplastic carcinomas,3 squamous carcinomas,3 cistadenenomas, 3 adenomas ,3 papillary adenomas,3 fibroadenomas, 3 cystic papillary fibroadenomas

Cuts were 5 mm thick and were processed by the following identification methods: Picrosirius and polarization microscopy for collagenous fibers[10;3] method for reticular fibers[24] fucsine-resorcine method for elastic fibers.

RESULTS

Collagen system: The use of the Picrossirius method in the histological cuts of benign neoplasms and carcinomas enabled a clear visualization of collagen as thick fibers irregularly distributed in the stroma of carcinomas and in a more orderly and regular fashion in benign neoplasms.A smaller amount of thin fibers was observed in an irregular and random disposition in carcinomas and in a regular disposition in benign neoplasms. Few differences in collagen population had been observed among the histologic types

of carcinomas and among types of benign neoplasias.

Carcinomas present a collagen population mainly composed of thick, irregular and randomly distributed fibers in its stoma, which are more evident in the central hyaline regions. These fibers are disposed as large bands that separate groups of tumoral cells or form nests with few neoplastic cells. They frequently surround partially or discontinuously the neoplastic area. Thick and intensely colored fibers often accumulated in the apical top were seen around the papillae, directed internally. Around of papillae and internally in the apical top, thick and intensely colored fibers, often accumulated. The solid areas were formed by dense stroma cut by long fibers ; anastomosed and intensely colored.The anaplastic areas presented few fine and long fibers larded by thick and short fibers in a irregular and randomly disposition, and many times forming rosettes .

Benign neoplasm :Thick collagenous fibers forming bundles and in a regular disposition are found around the neoplastic area. Fibers surround this area completely. The stroma present a few fine and long fibers, larded by thick and short fibers, anastomosed and in a parallel disposition. Neighboring the papillae region, the stroma was denser and regular, intensely colored fibers were observed in the internal part of the papillae.

Under polarization :

Carcinomas: Thick collagenous fibers forming bundles and in a parallel disposition were found around the neoplasic area, in a discontinuous fashion. Fibers present different lengths, were yellowish or reddish and strongly birefringent, what suggests that they were collagen type I. In the middle of these fibers, other ones, pale, greenish and weakly birefringent, some of them thinner, possibly collagen type III were observed. In the stroma, fibers were mostly thick, strongly birefringent, yellowish or reddish, disposed in an irregular and random fashion, mainly in the central areas, many times forming large bands which separate groups of tumoral cells. Around tubules and acini and in the neighboring regions, some thinner, pale and greenish, weakly birefringent fibers may be observed.

Benign neoplasms: Thick collagenous fibers forming bundles and in an regular disposition were found around the neoplasic area. Fibers were yellowish or reddish and strongly birefringent, what suggests that they were collagen type I. In the middle of these fibers, other ones, pale, greenish and weakly birefringent, some of them thinner, possibly collagen type III. In the stroma, fibers were mostly thick, strongly birefringent, yellowish or reddish, what makes the tumoral mass present a lobular aspect. Around tubules and acini and in the neighboring regions, some thinner, pale and greenish, weakly birefringent fibers may be observed, which were probably collagen type III.

In the observation of condrometaplasic areas, both in malignant and benign neoplasms, there was a collagen population composed by thin fibers in a parallel disposition, limiting narrow regions where condrocytes were aligned. Around this area, there was a collagen population formed by bundles of thick anastomosed fibers, irregularly disposed in carcinomas and orderly, in a parallel fashion in benign neoplasms (Fig. 1, 2, 4 and 5).

Reticular fibers

The use of reticular fibers staining by Gordon & Sweats, in the histological cuts of benign neoplasms and carcinomas ,enabled a clear visualization of collagen as thin fibers disposed not only in the dense stromas but also in the loose ones. These fibers presented variable density, but were found mainly around acini and tubules (Fig. 3).

Elastic system

Weigert's method with or without oxidation enabled the visualization of the fibers in the elastic system. Although no morphometric evaluation was performed, when elastic fibers - elauninic and oxytalan - were compwered to reticular and collagen fibers, it may be observed that the elastic system is less representative in relation to other matrix elements.

In relation to the presence elements of the elastic system in benign and malignant tumors, it may be observed that they predominate in the malignant ones, mainly in the pseudocapsule and around acini and tubules. Elements of the elastic system were not observed in the specimens when they were submitted to Weigert's staining without oxidation. When the same material was submitted to Weigert's staining with oxidation for evidencing of oxytalan fibers, these elements were more evidently seen around acini and tubules, as well as in the pseudocapsule.

Besides acini, tubules and the pseudocapsule, elements of the elastic system were in the ECM, both in samples submitted to staining with oxidation and without it. This observation is similar for benign and malignant tumors.

In anaplastic carcinomas (with low level of differentiation), elastic fibers were scant not only in the pseudocapsule, but also in acini and tubules. In relation to the other histological types studied, and after oxidation, a larger amount of oxytalan fibers were seen.

In the samples of cartilaginous and bone metaplasies, no elements from the elastic system could be seen when staining by Weigert's with or without oxidation was performed (Fig. 6).

DISCUSSION AND CONCLUSIONS

Several aspects of neoplasms have been largely studied. The study of the extracellular matrix in benign and malignant tumors is comparatively scant, in spite of its

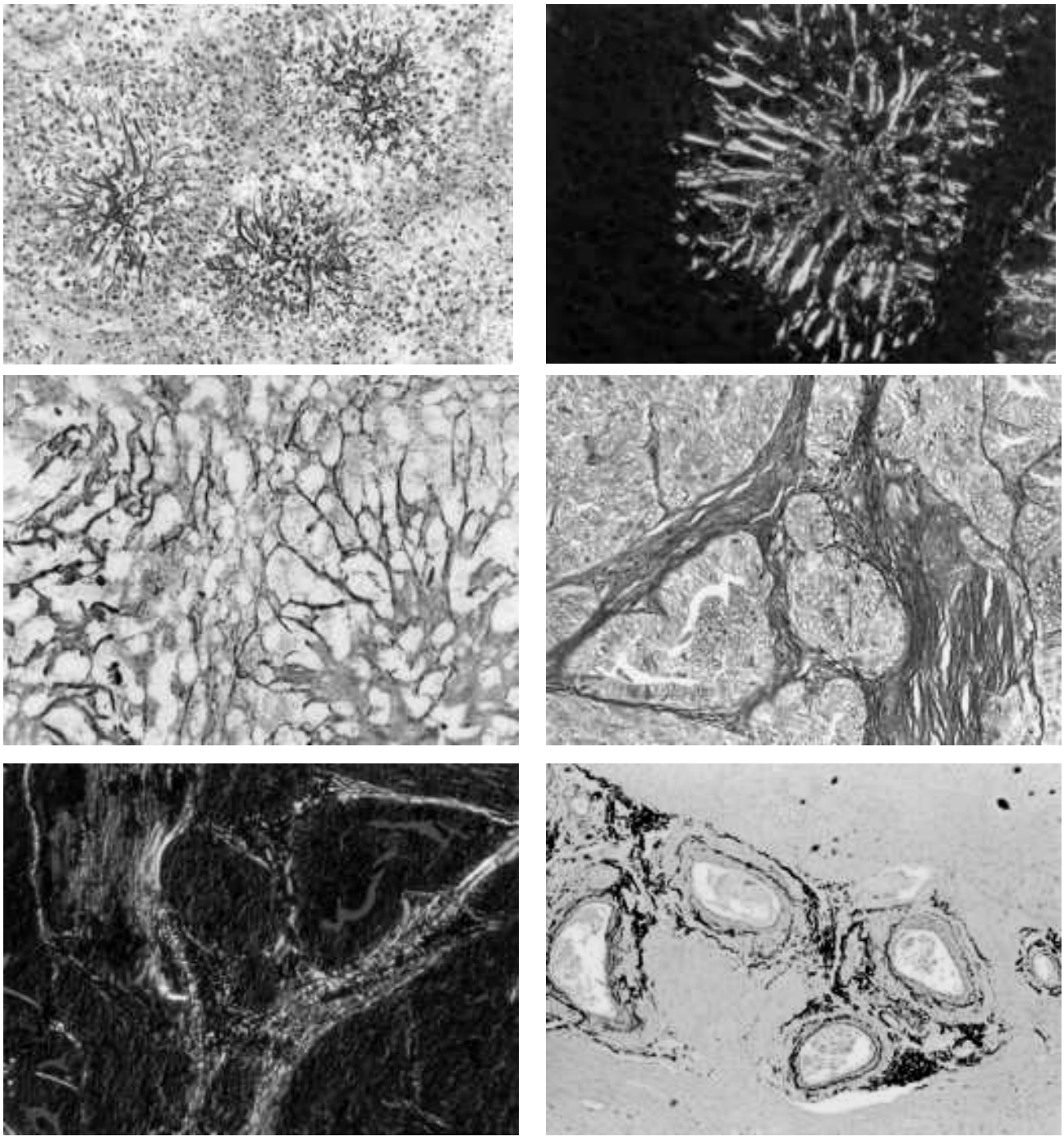

Figure 1 - Photomicrography of anaplastic carcinoma, evidencing areas showing bone metaplasia. Picrosirius method. Magnification: 34 x. **Figure 2 -** Detail of the previous photomicrography showing bone metaplasia under polarized light. Type I collagen fibers may be observed in white. Picrosirius method. Magnification: 140 x. **Figure 3 -** Photomicrography of anaplastic carcinoma (same neoplasm of the previous photomicrography), stained by silver. Reticular fibers may be seen in black. Gordon & Sweet staining method. Magnification:140 x. **Figure 4**- Photomicrography of solid adenocarcinoma, showing the distribution of the extracellular matrix. Picrosirius method. Magnification: 140 x. **Figure 5** - Photomicrography of solid adenocarcinoma under polarized light. Type I collagen in whitish.. Picrosirius method. Magnification: 140 x. **Figure 6 -** Photomicrography of adenoma, stained by Weigert without oxidation, showing the fibers of the elastic system, mainly around the vessels.Magnification: 140 x.

importance. This was a great stimulus for the performance of this trial, using histochemical methods.

Results presented here show the variability in the expression of ECM in relation to stroma component studied in carcinomas and benign neoplasms. Carcinomas present a collagen population mainly composed of thick, irregular and randomly distributed fibers in its stroma, which were more evident in the central hyaline regions. This fibers were disposed as large bands that separate groups of tumoral cells or form nests with few neoplasic cells; they frequently surround partially or discontinuously the neoplasic area. Benign neoplasms presented fibers disposed regularly which give it a lobular aspect. Fibers surround the neoplasic area completely. Under polarization, these fibers were seen as thick, strongly birefringent and yellow-reddish, which is suggestive of collagen type I. It was also possible to observe that, both in carcinomas and benign neoplasms, there was a small population formed by thinner fibers, weakly birefringent, pale and greenish, mainly around tubules and their neighboring region, what is suggestive of collagen type III pattern. These finding were in agreement with those by[14], and with finding for other kinds of tumors (neurinomas, neurofibromas, fibromas, osteosarcomas)[9,12].

In the observation of condrometaplasic areas, both in malignant and benign neoplasms, there was a collagen population composed by thin fibers in a parallel disposition, limiting narrow regions where condrocytes were aligned. Around this area, there was a collagen population formed by bundles of thick anastomosed fibers, irregularly disposed in carcinomas and orderly, in a parallel fashion in benign neoplasms. Picrossirius method together with polarization demonstrated that this population, among condrocytes, was formed by weakly birefringent fibers, pale and yellowish, what suggests a collagen type II pattern. In the surrounding layer, there was a population of strongly birefringent fibers, what is suggestive of collagen type I. Collagen type II is an important component of hyaline and fibrous cartilage; it is, however, difficult to be seen in the optical microscope, for fibers were very small and difficult to be stained due to the intense association with proteoglycans[10,11,25,18].

The presence of thin argirophylic fibers (typical reticular fibers) was evidenced by silver impregnation. These fibers form a supporting net around the tubules in the neoplasms, but they present a more irregular arrangement in carcinomas. The correspondence between reticular fibers and the thinner, pale, greenish and weakly birefringent fibers of collagen III, evidenced by the Picrossirius associated to polarization method and by the silver impregnation technique was easily visualized, what is in accordance with data found in the specialized literature, suggesting that reticular fibers were constituted of collagen type III[20,2].

Because of the complexity of the elastic system and the considerable number of histological variables in benign and malignant neoplasms, as well as the diversity of matrix elements in the stroma of these neoplasms, the discussion will be divided in categories of neoplasms, their level of differentiation and the presence of cartilaginous and bone metaplasia.

Benign tumors presented less elements of the elastic system, mainly around tubules and acini, even when the specimens were submitted to oxidation. On the other hand, malignant tumors, even the anaplastic ones, presented more of these elements, when submitted to oxidation. It should be emphasized that, both in the benign and the malignant tumors it was almost impossible to observe elastic fibers, mainly around tubules and acini.

Based on these results, it may be stated that oxytalan fibers were the predominant ones in these well-defined histological regions. Although reports on mammary tumors in dogs were scant or non-existent, these finding make sense, when the elastosis or periductal hyperplasia described in mammary human neoplasms were considered [16,4] .

There was almost total absence of fibers from the elastic system in the ECM, except in the periacinar and peritubular regions. This finding was similar for benign and malignant tumors. No data was found in the specialized literature in relation to this aspect in mammary tumors in dogs.

The pseudocapsule of the neoplasms studied were rich in elements. Both in the benign and malignant neoplasms submitted to oxidation in Weigert's method and those submitted to the staining without oxidation presented a solid arrangement similar to a string in the pseudocapsule. This morphological aspect may be related to the development and invasive power of the neoplasm, depending on its level of differentiation. There were reports in the specialized literature related to its function in limiting distention and preventing the rupture of the capsule[22]. The findings presented here, together with the importance of these arrangements of the elements of the elastic system with the collagen fibers in the pseudocapsule enable to infer the importance of the pseudocapsule in the restraining and invasive power of neoplasms.

The presence of mainly oxytalan fibers in the peritubular and periacinar regions almost invariably observed in these structures seem to serve as an anchor or connection for these elements with macromolecules in the stroma of the neoplasm. Another evidence in favor of this hypothesis is the presence of reticular or collagen type III fibers in this region.

The larger quantity of these fibers in carcinomas may be due to the larger rate of matrix degradation by malignant tumoral cells. Because of this, a larger quantity of oxytalan than of collagenous - collagen type III - reticular fibers would be formed in the matrix of these tumors[6]. According to this author, there is no relationship between the increase in the number of oxytalam fibers and tumor progression.

No fiber of the elastic system was observed in the study of the elastic system in the cartilage of neoplasms

ECM in specimens stained by Weigert's method with or without oxidation. These findings were not in accordance with the systematic study of these fibers in fibrous and hyaline cartilage performed by[19]. This author demonstrated the presence of oxytalan fibers in the hyaline cartilage in the ECM around condrocytes; elastic fibers located in the fibrous layer of the pericondrium and oxytalan and elauninic fibers in fibrocartilage.

Although many macromolecules present in the extracellular matrix should be studied, results obtained here should be used as prognosis values in studies of groups of patients and may incentive new studied in order to achieve a wide characterization of the elements that form the extracellular matrix in mammary neoplasms in dogs.

RESUMO

A finalidade do presente trabalho foi estudar algumas das proteínas fibrilares da matriz extracelular de 54 neoplasias mamárias benignas e malignas na espécie canina, utilizando métodos histoquímicos: Picrosirius associado à polarização para fibras colágenas , método de Gordon - Sweats para fibras reticulares e método de Weigert com e sem oxidação para fibras elásticas. Evidenciou-se na matriz uma grande variabilidade na quantidade, distribuição e características dos componentes matriciais presentes nos diferentes tipos de neoplasias. Detectou-se, assim, colágeno I, III e elementos do sistema elástico, distribuídos diferentemente nas neoplasias benignas e malignas. O método Picrosirius simples e associado à polarização permitiu a visualização do colágeno sob a forma de fibras espessas distribuídas irregularmente no estroma dos carcinomas e de modo mais ordenado e regular nas neoplasias benignas e, fibras mais finas, em menor quantidade, irregularmente e aleatoriamente dispostas nos carcinomas e regularmente nas neoplasias benigna. Sob luz polarizada os feixes de fibras colágenas , apresentaram diferentes comprimentos, avermelhados ou amarelados e fortemente birrefringentes, sugerindo serem colágeno tipo I e, entremeando as fibras, algumas mais finas ,pálidas, esverdeadas e fracamente birrefringentes, presumivelmente colágeno tipo III. Em áreas condrometaplásicas, tanto nos carcinomas como nas neoplasias benignas notou-se que os feixes colágenos apresentavam-se com fibras finas, paralelas, limitando regiões estreitas onde os condrócitos se aninhavam, e, rodeando esta área, feixes de fibras espessas, anastomosadas, dispostas irregularmente nos carcinomas e ordenadamente e paralelas nas neoplasias benignas. Sob luz polarizada, essa população entre condrócitos era formada por fibras pálidas e amareladas, sugerindo padrão tipo II e na faixa circundante, feixes fortemente birrefringentes, sugerindo o padrão do colágeno tipo I.. O uso do método - Gordon & Sweats, permitiu a visualização do colágeno sob a forma de fibras finas, dispostas tanto nos estromas densos como nos frouxos, com densidade variada mas, principalmente , margeando os ácinos e túbulos. Quanto à presença do sistema elástico em tumores benignos e malignos, verificou-se que há predomínio dessas fibras nos malignos, principalmente na pseudocápsula e ao redor de ácinos e túbulos. Elementos do sistema elástico não foram observados em espécimes submetidos à coloração de Weigert sem oxidação. Neste mesmo material, com oxidação, as fibras oxitalâmicas foram mais evidentes principalmente ao redor de ácinos, túbulos e pseudocápsula. Elementos do sistema elástico na (MEC) foram raros, tanto em amostras com e sem oxidação. Isto pode ser observado de maneira semelhante tanto em tumores benignos e malignos. Os resultados deste estudo enfatizam as profundas alterações estruturais dos componentes colagenosos e fibras do sistema elástico na matriz extracelular de neoplasias mamárias da espécie canina.

PALAVRAS-CHAVE: Neoplasias mamárias. Colágeno. Cães. Matriz extracelular.

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