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Apoptosis as a prognostic marker in canine mammary tumors by TUNEL

A apoptose como marcador prognóstico em tumores mamários caninos pelo método TUNEL

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Summary

Apoptosis plays an important role in oncogenesis determining tumor growth and aggressiveness. The aim of this study was to examine the relationship between apoptosis, diagnosis and prognosis in canine mammary tumors. Thirty bitches were submitted to tumor extirpation and a method of labeling apoptotic cells, that labels DNA fragments, by terminal deoxynucleotidyl-tranferase (TdT) mediated by 5' deoxy-uridine-triphosphate (dUTP) nick and labeling (TUNEL) was used. The apoptotic cells were counted in ten high power fields (10HPF). Statistical results showed that when there are more than 30 apoptotic cells/10HPF the prognosis is worse (p= 0,0005). Thus suggesting that apoptosis can be used as a prognostic marker in canine breast tumors.

Key-words
Apoptosis.
Prognosis.
Breast cancer.
Bitch.

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Introduction

Recently there has been a special interest shown in the study of bitch mammary gland tumors due to their similarity with human breast cancer[1,2,3]. Furthermore, the mammary gland is the most commonly site to be affected by neoplasias in bitches representing 50.00% of all tumors in this species[3,4,5,6,7,8,9].

Many studies attempt to clarify the mechanisms of carcinogenesis and advances in molecular biology and DNA cloning techniques have helped in a better understanding of the complex events which occur during the malignant transformation of tumors[10]. With this there is hope to develop new cancer treatments[11,12,13] in order to improve the prognosis of individuals suffering from cancer [10,14,15].

Several tumors present activated genes which are not normally expressed in the adult tissue from which they originate. The proteins derived from the expression of these genes are called tumor markers, and the detection and measurement of these proteins are used in medicine for diagnosis and assessing the evolution of the tumors[16]. Much effort has been made to develop objective methods to evaluate cellular proliferation and its relation to the prognosis[17, 18].

Recently, molecular alterations, which are observed in the cells of cancer patients, started to be understood and they are linked to the mechanisms which regulate normal cell division, survival and cell death[19]. Programmed cell death, also known

as apoptosis, plays an important role in the determination of tumor growth and its aggressiveness[20]. Thus, apoptosis has been considered as a inherent and regulating cellular mechanism, through which there is a control between the production of new cells and the individual capacity of the self-destruction of the cells[21,22,23,24]. An understanding of this process is of fundamental importance for man as homeostasis does not only depend on the ability of the organism to produce new cells, but also the individual ability of the cells to destroy themselves when they become superfluous or uncoordinated [10,19].

The beginning of the process is rigidly controlled by numerous intracellular and extracellular signals capable of inducing programmed cell death[12, 24]. This cellular suicide involves specific proteases, called caspases, which are activated by the proteolytic cleavage as a response to signals which induce programmed cell death. These active proteases cleave key proteins from the cells and kill them rapidly and orderly[19]. Regulation of this process is as complex as the regulation of cellular growth and it accompanies a series of biochemical alterations and morphologic characteristics[12], with morphological alterations of the cell and the nucleus[25].

In relation to cancer, some researchers still do not believe that determination of the apoptosis rate can be utilized as an independent prognostic indicator[26], but another group demonstrated that the estimation of apoptosis can be used as such, similar to the cellular proliferation rate and expression of the bcl-2 or p53 proteins[20]. Therefore, they believe that measurement of cellular proliferation and apoptosis may improve the reliability of the prognosis of tumoral behavior[27] and its utilization might biologically characterize the tumor[28], as this phenomenon is fundamental in oncogenesis[27].

Apoptosis can be detected by microscopy, conventional histopathology, or even evidenced by special techniques[29]. A method which labels fragments of DNA and which has frequently been used, is that of terminal deoxynucleotidyl-tranferase (TdT) mediated by 5' deoxy-uridine-triphosphate (dUTP). This method labels the 3'-OH ends of DNA fragments and is known as TUNEL[30]. The 3'-OH ends resulting from DNA fragmentation, are labeled with nucleotides modified by the TdT enzyme. This enzyme is more selective in detecting apoptosis than necrotic cells[31]. The apoptotic cell count can be considered as a relation of the percentage of the tumoral cells present, that is, as the number of apoptotic cells per square millimeter of neoplastic tissue, and it is usually designated as the apoptotic index (AI)[29].

Some researchers consider that, when the AI is high, the tumor grows at a slower rate and when it is low, the tumor grows rapidly[21]. But taking into consideration that during tumoral growth there is an equilibrium between the proliferation and cellular death, in which the process of apoptosis is involved[32], the high number of apoptotic cells will be proportional to a high cellular proliferation rate and an unfavorable prognosis[27]. According to one study[17], the survival of patients with mammary carcinomas who have 30 or more apoptotic cells per 10 high power fields (10HPF), was greater than patients with a count of less than 30 cells per 10HPF.

In this way, considering the importance of apoptosis in neoplasias and the lack of understanding in relation to its real function in mammary tumors in bitches, this study aimed to verify the expression of the apoptotic markers by the TUNEL method. Its

study in relation to the tumoral malignancy showed that the evolution of these tumors can be predicted, and so this can be a very useful tool in the determination of prognosis of this type of tumor which otherwise is difficult to classify and diagnose.

Material and Method

A total of 30 sections of neoplastic tissues from bitches that were attended at the "Dr. Halim Atique" – UNIRP Veterinary School were submitted to surgical ablation of their tumors during the period of February to May 2001. The tumoral fragments, conserved in formalin and embedded in paraffin, underwent sectioning with 5 mm of thickness, where one was dyed with hematoxylin e eosin (HE) for histopathologic diagnosis and another was submitted to the TUNEL technique for the detection of apoptosis. In order to assess the prognosis, the bitches were accompanied for up to six months after the surgical ablation to observe the presence of re-incidence or metastasis. Additionally, the time of evolution and the macroscopic characteristics were analyzed according to the TNM classification[1], as well as post-operative response and death.

TUNEL technique – The kit used was the Apoptag® Plus Peroxidase *in situ* Apoptosis detection Kit (Intergen® company Discovery products ™). Labeling of the 3'-OH fragment ends was achieved by TdT mediated using dUTP. The sections cut from paraffin blocks were removed from the paraffin, hydrated and rinsed in distilled water. Subsequently, the sections were covered by a quantity of 100ml of proteinase K (diluted in the proportion of 20 mg/ml of distilled water) and placed for seven minutes in an incubator at room temperature. After the sections were washed in distilled water (4 times of 2 minutes each) and immersed in Phosphate buffer solution (PBS) for 3 minutes. These preparations were then immersed in 3.00% hydrogen peroxide solution for 15 minutes and after washed with PBS (2 times for 5 minutes each time) and again put into the incubator at room temperature for 20 minutes in an equilibration buffer solution. After removing the excess they were put with 26.88 ml of a solution comprised of 6.4 ml of TdT solution with16 ml of reaction buffer and 4.48 ml bovine serum albumin on a slide and subsequently left in a humid chamber at 37° C for 1 hour. Following this, the material was steeped in stop wash solution (1 ml/34 ml of distilled water) for 20 minutes and after washed with PBS for 3 times of 5 minutes each time. Anti-digoxygenin was put at a rate of 30 ml over the sections for 30 minutes and again they were washed in PBS (4 times for 5 minutes each). Diluted diaminobenzidine (DAB) solution (117 ml of "DAB dilution buffer" and 13 ml of "DAB substrate") was used at the rate of 26 ml per slide and left for 5 to 10 minutes. Thereafter the slides were washed in running water for 5 minutes and rinsed in distilled water (3 times for 1 minute each). After this counter-staining using Harris' Hematoxylin was performed for 10 seconds, the sections were washed in running water and afterwards they were rapidly dipped in 0.50% ammonia hydroxide solution and left in running water again for 7 minutes. Finally the slides were dehydrated and mounted in resin. The TUNEL technique reaction was demonstrated by the presence of a golden-brown coloration which showed the specific morphological characteristics of apoptosis [33].

Results

The histopathologic diagnostic results of the study group demonstrated that Malignant Mixed Tumors were the most commonly observed totaling 47.00% of the total number of cases followed by carcinomas with a

frequency of 40.00%. Benign Mixed Tumors were only seen in 13.00% of the canine mammary neoplasias, as is indicated in Figure 1.

According to Table 1, a relation among the histopathologic diagnosis, prognosis and the apoptotic rate of tumors in this group was observed. Statistically there was not a positive correlation between the clinical diagnosis and the prognosis of the tumors. The same situation was seen when the histopathologic diagnosis was compared to the number of apoptotic cells per 10HPF, showing that the correlation among these data was not significant.

Apoptosis labeling was expressed as a number of apoptotic cells in 10 high power felds, considering the value to be higher or lower than 30 apoptotic cells per field. The labeling of the apoptotic cells was demonstrated by a golden-brown coloring[33] as can be seen in Figures 2 and 3 which were analyzed in pre-determined areas of the histological preparations. These areas with a characteristic tumoral aspect were split into 10 fields in the lens at a magnification of 40 X[20].

Statistically the results were evaluated to clarify the true value of these prognostic markers in canine mammary neoplasias. Thus, the results obtained from the labeling of apoptosis, by a similar method to Gonzáles – Cámpora[20], were analyzed and compared to the prognosis of the animal (Figure 4).

The number of apoptotic cells was compared to the prognosis using the Exact Fischer Test. This test is used when the available samples are small. It can be seen that the possibility of a poor prognosis for the members of the group was higher when the apoptosis rate was also seen to be higher (p value = 0.0005). According to these data, it was verified that all of the bitches with more than 30 apoptotic cells per 10HPF showed a poor prognosis and not one of them possessed a rate less then 30 apoptotic cells per 10HPF.

Discussion and Conclusion

Apoptosis can be considered a favorable prognostic marker for individuals with breast neoplasias as,

Figure 1
Histopathologic diagnosis of tumors in bitches attended in UNIRP´s Veterinary Hospital from February to May, 2001

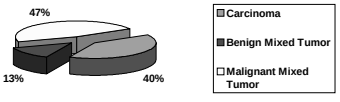
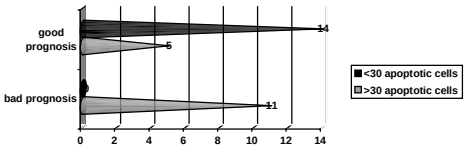


Figure 4
Results of apoptosis labeling of canine mammary neoplasias in relation to the prognosis of bitches attended in UNIRP´s Veterinary Hospital from February to May 2001



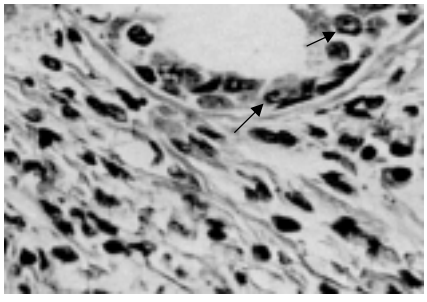


Figure 2
Photomicrography of a Malignant Mixed Tumor (40X), indicating the apoptotic cells in a golden-brown (> 30 apoptotic cells/10HPF)

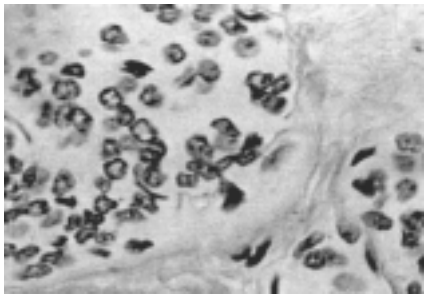


Figure 3
Photomicrography of a Benign Mixed Tumor (40X), negative for the apoptotic cells (< 30 apoptotic cells/10HPF)

Table 1
Diagnosis, apoptotic rate and prognosis of bitches attended in UNIRP´s Veterinary Hospital from February to May, 2001

	Histopathologic Diagnosis	Apoptosis Index (cells/10HPF)	Prognotic
1	Benign Mixed Tumor	< 30	Good
2	Solid Carcinoma	> 30	Bad
3	Malignant Mixed Tumor	< 30	Good
4	Benign Mixed Tumor	> 30	Bad
5	Malignant Mixed Tumor	< 30	Good
6	Benign Mixed Tumor	< 30	Good
7	Malignant Mixed Tumor	< 30	Good
8	Malignant Mixed Tumor	> 30	Bad
9	Malignant Mixed Tumor	> 30	Bad
10	Malignant Mixed Tumor	< 30	Good
11	Malignant Mixed Tumor	> 30	Good
12	Malignant Mixed Tumor	> 30	Good
13	Tubulo-alveolar Carcinoma	< 30	Good
14	Solid Carcinoma	> 30	Bad
15	Malignant Mixed Tumor	< 30	Good
16	Malignant Mixed Tumor	> 30	Bad
17	Malignant Mixed Tumor	> 30	Good
18	Malignant Mixed Tumor	> 30	Good
19	Malignant Mixed Tumor	> 30	Good
20	Solid Carcinoma	< 30	Good
21	Benign Mixed Tumor	< 30	Good
22	Squirrous Carcinoma	< 30	Good
23	Malignant Mixed Tumor	< 30	Good
24	Medular Carcinoma	> 30	Bad
25	Tubulo-alveolar Carcinoma	> 30	Bad
26	Intra-ductal Carcinoma	> 30	Bad
27	Tubulo-alveolar Carcinoma	< 30	Good
28	Intra-ductal Carcinoma	< 30	Good
29	Medular Carcinoma	> 30	Bad
30	Medular Carcinoma	> 30	Bad

according to reviewed works[10,19], programmed cell death controls homeostasis stabilizing the growth and impeding tumoral evolution. But, as opposed to this, some researchers report that during tumoral growth there is an equilibrium between the proliferation and programmed cell death in which the apoptosis process is involved[32]. If the presence of a greater number of apoptotically marked cells is related to a high rate of cellular proliferation, this consequently reflects on a less favorable prognosis for this individual[27]. Hence, it can be inferred that individuals with a greater number of cells labeled by apoptosis has a survival rate lower than those with a lower rate of cells labeled by apoptosis[20].

Furthermore, considering that, individuals with neoplasias present a high cellular proliferation rate and that, so that homeostasis occurs, the control is achieved by the process termed apoptosis, which controls the tumoral cell population. It is concluded that the higher the number of apoptotic cells the higher the cellular proliferation, and with this, of course, the tumoral aggressiveness.

The results obtained in this study showed that statistical significance was detected between apoptosis and prognosis[20], which means that when a higher number of apoptotic cells is observed present in neoplasias, a worse prognosis must be expected (p value = 0.0005).

According to the obtained results, in agreement with other researchers[20, 27], who also correlated a higher number of cells labeled by apoptosis to the high rate of cellular proliferation, which is responsible for the tumoral growth. Therefore, labeling

of apoptosis by the TUNEL method proved to be statistically significant in relation to a poor prognosis in individuals who have mammary neoplasias, and thus it can be considered a good marker, thus improving the knowledge and predicting the life expectancy of cancer patients.

Resumo

A apoptose, como evento celular, tem uma participação importante na tumorigênese, determinando o crescimento tumoral e sua agressividade. O presente estudo, teve como objetivo, examinar a relação entre a apoptose, o diagnóstico histopatológico e o prognóstico na neoplasia mamária canina. Trinta cadelas foram submetidas a exérese tumoral e o fragmento tumoral submetido ao método de marcação das células apoptóticas, que marca fragmentos de DNA da célula em apoptose, conhecido como desoxinucleotídeo terminal transferase (TdT) mediado pela 5' desoxiuridina trifosfato (dUTP) ou TUNEL. As células em apoptose foram contadas em 10 campos de maior aumento (10HPF) sendo que os resultados estatísticos demonstraram uma correlação positiva entre a apoptose e o prognóstico ruim (p= 0,0005). Dessa forma, a apoptose pode ser considerada um marcador prognóstico nas neoplasias mamárias caninas.

Palavras-chave
Apoptose.
Prognóstico.
Neoplasias mamárias.
Cadelas.

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