

CONTAGEM DE CÉLULAS CONJUNTIVAS POR CITOLOGIA DE IMPRESSÃO EM BOVINOS E EQUINOS

COUNTING OF CONJUNTIVAL CELLS BY IMPRESSION CYTOLOGY IN BOVINE AND EQUINE

Roberto C. Gonçalves¹, Eric C. Pereira¹, Luis M. Montoya^{1,2}, Francisco J. Pedraza^{1,2}, Simone B. Chiacchio¹, Rogério M. Amorim¹, Alexandre S. Borges¹, Noeme S. Rocha^{1*}

¹Department of Veterinary Clinics, School of Veterinary Medicine and Animal Science, Universidade do Estado de São Paulo, Botucatu, Sao Paulo, Brazil

²Veterinary Pathology Research Group, Faculty of Agricultural Sciences, Universidad de Caldas, Manizales, Colombia

rochanoeme@fmvz.unesp.br

Resumo: A citologia por impressão é um método não invasivo útil para avaliar a superfície ocular. Consiste na aplicação de um fragmento de papel filtro sobre a conjuntiva. As amostras obtidas podem ter até três camadas de células e são distendidas em lâminas histológicas. O estudo teve como objetivo avaliar o padrão qualitativo e quantitativo das células da conjuntiva bulbar em bovinos e cavalos clinicamente saudáveis para facilitar o diagnóstico de doenças oculares externas. Para este fim, foram coletadas amostras de células externas da conjuntiva ocular pelo método de citologia de impressão de 15 bovinos e 15 equinos adultos. Para o exame citológico as amostras foram coradas por Giemsa e Shorr. Duas lâminas foram preparadas de cada animal, uma do olho esquerdo e outro do olho direito e foram contadas 800 células em total. Para a análise estatística, o ANOVA foi utilizado, foram considerados significativos os valores de $p < 0,05$. A análise permitiu estabelecer diferenças no número de células caliciformes, linfócitos e eritrócitos entre espécies e animais. A citologia por impressão da conjuntiva bulbar foi eficaz na análise celular e foi bem aceita pelos bovinos e equinos.

PALAVRAS-CHAVE: citologia, oftalmologia, bovino, equino

Abstract: Impression cytology is a useful, non-invasive method of investigating the ocular surface. This method consists of applying a piece of filter paper to the conjunctiva. The samples obtained can comprise up to three cell layers, which are applied to a histological slide. This study aimed to evaluate the quality and quantity of cells in the bulbar conjunctivas of clinically healthy cattle and horses to facilitate the diagnosis of external eye diseases. To this end, samples of external ocular conjunctiva cells were collected from 15 adult cattle and 15 adult horses using the impression cytology method. For cytology, the samples were stained with Giemsa and Shorr. Two slides were prepared for each animal, one from the left eye and one from the right eye, and 800 cells were counted in total. For the statistical analysis, ANOVA was used with $p < 0.05$ considered to be statistically significant. The analyses allowed for the comparison of the numbers of goblet cells, lymphocytes, and erythrocytes between the species and among the sampled animals. Impression cytology of the ocular surface was effective, and the technique was well accepted by cattle and horses.

KEY-WORDS: cytology, ophthalmology, cattle, horses.

INTRODUCTION

Due to its superficial location, the eye is exposed to a wide range of microorganisms, toxic chemical compounds and antigens, in

addition to solar radiation and adverse climatic conditions, making it vulnerable to a large variety of lesions. Although the reactions of the eye to injurious agents are basically identical to those that occur in

other parts of the body, they deserve special attention because the eye is affected in numerous systemic diseases and the recognition of ocular abnormalities aids in the diagnosis of many other conditions (Naib, 1972). Conjunctival lesions play an important role in the diagnosis of external ocular diseases (Naib, 1972). This structure is the main site of lesions of different etiologies, including degenerative changes, vascular diseases, inflammatory diseases and impairments in growth and development, for which differential diagnosis by clinical examination alone may be inaccurate (Lima et al., 2005).

For the examination of the effects of these disorders on the conjunctiva, several cell sampling techniques have been employed, which vary according to the localization of the lesions (Rosenthal et al., 1997; Font et al., 2001). Exfoliative abrasive aspiration and impression cytology are two techniques that are used for conjunctival evaluation. These methods provide material in adequate amounts for evaluation, they preserve the morphological characteristics of the tissue, and they are not uncomfortable or painful for the patient (Bolzan et al., 2005). Impression cytology is a method for obtaining cells of the conjunctival and corneal epithelia (Egbert et al., 1977) and is often useful in the diagnostic phase before conducting other more invasive or more expensive procedures (Raskin & Meyer, 2003; Rito, 2009). This method allows the easy collection of study material, it is non-invasive, and minimal trauma is induced (Barros et al., 2001). It is especially useful in the diagnosis of conjunctivitis and keratitis (Gilger, 2006). This technique is an alternative to excisional biopsy and cytology smears obtained from the ocular surface, assuring a better quality of the samples (Dart, 1997).

The impression cytology was developed based on the discovery by Egbert et al. (1977) that cells of the epithelial layer could be removed by applying a filter paper composed of cellulose acetate to the ocular surface, and it has been used to evaluate the epithelium of the ocular surface in a variety

of conditions (Tseng, 1985; Maskin & Bode, 1986; Nolan et al., 1994). This technique has been substantially improved by several researchers (Anshu et al., 2001).

The impression technique provides superior data with respect to the anatomical localization of the conjunctival and bulbous cells, the cell-to-cell ratio and the interaction among the epithelial cells as well as among other cellular components (Naib et al., 1967; Tseng, 1985; Nolan et al., 1994; Yagmur et al., 1997). In addition to being a painless technique that is well tolerated by patients, impression cytology yields good cellularity with excellent cellular details and preservation of the cellular morphology (Yagmur et al., 1997; Anshu et al., 2001). Despite the clear advantages of impression cytology, this method is rarely used, especially in veterinary medicine.

The technique consists of applying a filter paper made of either cellulose acetate or cellulose nitrate (Anshu et al., 2001) onto the ocular surface. According to the literature, Millipore filters offer the best results (Nolan et al., 1994; Anshu et al., 2001; Barros et al., 2001). The pore size of the most frequently used filter paper is 0.45 μm , and this size may vary from 0.22 to 0.45 μm (Barros et al., 2001). This filter is pre-cut into strips with a form that enables an impression in any quadrant of the ocular surface, maintaining its orientation on all slides.

Before application, a topical anaesthetic such as xylocaine 4% (Anshu et al., 2001) can be used; however, certain anaesthetics may alter the cellular morphology and interfere with the evaluation. Therefore, anaesthetics are not used except in cases of intolerable discomfort of the patient (Yagmur et al., 1997). The strips are pressed for 3 to 5 seconds onto the conjunctiva with a stick or tongs with a flattened end and then gently massaged. Each strip is then removed smoothly, together with 1 to 3 cell layers that are next placed onto a glass slide to be fixed and stained.

The morphological findings for normal cells of the cornea and conjunctiva in the horse are similar to those described for dogs and

cats (Murphy, 1988; Prasse & Winston, 1989). Thus, the most common findings from the cytological examination of the ocular surface are epithelial cells of the conjunctiva and cornea (superficial, intermediate and basal/parabasal); melanin granules; microorganisms; calciform cells and mucus; erythrocytes; and inflammatory cells, including neutrophils, lymphocytes and macrophages. Eosinophils and basophils are not normally present in corneal and conjunctival scrapings from horses (Giuliano *et al.*, 2002).

The present study sought to investigate the qualitative and quantitative patterns of cells from the eyes of clinically healthy cattle and horses to facilitate the future diagnosis of ocular diseases. It remains necessary to identify the cellular changes that occur in a variety of ocular afflictions and their correlation with normal standards.

MATERIALS AND METHODS

This study was approved by the Ethics Committee on Animal Experimentation of the Faculdade de Medicina Veterinária UNESP-Botucatu, São Paulo, Brazil. The samples were taken from the bulbar conjunctivas of 15 adult cattle and 15 adult horses. The animals were clinically healthy and had no history of eye disease. The animals were selected independently of breed, sex and age, and they originated from the School of Veterinary Medicine and Animal Husbandry - UNESP at Botucatu or from rural properties in the region around the city of Botucatu, São Paulo state, Brazil. After the mechanical containment of the animals, the cells were collected from the superior and inferior bulbar conjunctiva of the eye by impression with strips of Schleicher & Schuell filter paper (45 µm x 47 mm in diameter). These strips were pressed onto the conjunctiva by means of the index finger for 3 to 5 seconds. After this time, the paper strips were gently removed and pressed onto a glass slide to assess the deposition of the collected cells. The material from the superior zone of the eye

was deposited onto the upper portion of the slide, whereas that of the inferior zone was placed onto the lower portion of the slide. Two slides were prepared from each eye of each animal. The slides from each eye were fixed and stained, one according to the technique of Giemsa and the other by the method of Shorr.

The counting was performed using a common optical microscope at a magnification of 100x to observe the entire extension of the slide and evaluate the quality and quantity of the material. At 400x magnification, the cells were observed individually for the counting and differentiation of the epithelial cells (superficial, intermediate and basal/parabasal), calciform cells, erythrocytes and inflammatory cells (lymphocytes, neutrophils, macrophages and eosinophils). The counting standard was 200 cells for each ocular region (superior and inferior), corresponding to 400 cells per eye and 800 cells per animal at 400x magnification.

The material was analyzed, and the results were expressed as the total mean of the cells and the percentage of each cell type studied. For the statistical analysis, the ANOVA test was utilized, with $p < 0.05$ considered to be statistically significant.

RESULTS AND DISCUSSION

Cytology has been used in many medical applications as a method of auxiliary diagnosis and to gain information about the topography, the cellular pattern and the relationship between the epithelial cells and other cellular components (Tseng, 1985). In the present work, the technique of impression cytology removed a sufficient quantity of cells from the bulbar conjunctivas of the studied animals, thus enabling the identification and counting of the cell types present. In addition to being a simple, rapid, low-cost technique that is easy to execute, impression cytology was shown to be atraumatic and well accepted by both cattle and horses (Barros *et al.*, 2001). The

samples obtained possessed a high level of cellular preservation, and the methodology allowed for choosing the location for collection and establishing a ratio to describe the interaction between the epithelial cells and other cellular components (Naib et al., 1967; Tseng, 1985; Nolan et al., 1994; Yagmur et al., 1997). To collect the samples, only the mechanical stabilization of the animal was necessary. This requirement was considered to be an advantage over other methods that require the use of topical anaesthetics, as certain anaesthetics can interfere with the cell morphology and the evaluation (Yagmur et al., 1997; Anshu et al., 2001), although morphological alterations were not observed by Bolzan et al. (2005).

The results describing the means and percentages of epithelial cells, erythrocytes, inflammatory cells and the rates of cellular maturation in cattle and horses are presented in Tables 1 through 4.

The microscopic evaluation of the impressions showed epithelial cells from different conjunctival layers distributed as follows: 7.70% basal/parabasal, 20.51% intermediate and 61.51% superficial cells in cattle and 10.39% basal/parabasal, 23.58% intermediate and 61.58% superficial in horses. These proportions in the bulbar conjunctiva are similar to reports in dogs, for which a preponderance of superficial and intermediate cells has been described (Bolzan et al., 2005). These findings demonstrate the constant renewal of epithelial cells from the basal to the superficial layers to replace cells that have been injured or killed due to damage to the ocular surface, as well as normal cellular restoration/maturation.

Keratinized epithelial cells, present in 100% of the samples, were included in the group of superficial epithelial cells.

Occasionally, erythrocytes and inflammatory cells, such as neutrophils, lymphocytes, macrophages and eosinophils, have been reported in the conjunctival scrapings of normal horses (Giuliano et al., 2002). In the present study, we found that

lymphocytes corresponded to 7.20% of the cells collected from cattle and 2.5% of the cells collected from horses. Neutrophils corresponded to 2.0% of the cells from cattle and 0.7% of the cells from horses. Macrophages corresponded to 0% of the cells from cattle and 0.02% of the cells from horses. Eosinophils were not found in either cattle or horses.

The presence of inflammatory cells is normally attributed to at least one of the following causes: contamination of a sample with peripheral blood in the context of an invasive technique or improper performance of the sample collection technique, or inflammation depending on the presence or absence of clinical symptoms. Neutrophils are active in a variety of inflammatory responses, particularly in bacterial or fungal conjunctivitis. Lymphocytes are present in greater number in cases of viral conjunctivitis. Eosinophils become more numerous in cases of allergic conjunctivitis, a situation in which there is also greater shedding of epithelial cells. According to Giuliano et al. (2002), eosinophils and basophils are not normally found in conjunctival or corneal scrapings from horses, which is consistent with the results of the present work. Despite the quantities of inflammatory cells found in the present study, there were no clinical alterations of the ocular surface to indicate inflammation and/or apparent infection, such as conjunctival hyperaemia or secretions. These findings are consistent with those reported in the dog and cat, in which the presence of inflammatory cells in low numbers has been reported in healthy eyes (Bolzan et al., 2005).

The small quantities of erythrocytes present in the samples collected, corresponding to 0.4% in cattle and 0.1% in horses, demonstrate that impression cytology with filter paper is a minimally invasive technique that causes little or no injury during the process of collection from the ocular surface in the animals studied. In addition, this technique is well accepted by cattle and horses.

Table 1- Ocular conjunctiva cells from right and left eye of healthy cattle

Cells		Right eye				Left Eye			
		Superior		Inferior		Superior		Inferior	
		No	%	No	%	No	%	No	%
Epithelial	Superficial	122	61.2	103	51.3	131	65.6	136	67.9
	Intermediate	44	22.1	43	21.4	43	21.4	34	17.2
	Basal cells	17	8.7	22	11.2	12	5.9	10	5.0
Calciform cells		2	1.0	1	0.6	1	0.6	1	0.6
Leukocytes	Neutrophils	3	1.5	4	2.0	4	2.0	5	2.7
	Lymphoid Cells	10	5.2	26	12.8	9	4.4	13	6.4
	Eosinophil cells	0	0.0	0	0.0	0	0.0	0	0.0
Macrophages		0	0.0	0	0.0	0	0.0	0	0.0
Erythrocytes		1	0.4	1	0.7	0	0.1	0	0.2
	TOTAL	200	100.0	200	100.0	200	100.0	200	100.0

Table 2- Ocular conjunctiva cells from right and left eye of healthy horses

Cells		Right eye				Left Eye			
		Superior		Inferior		Superior		Inferior	
		No	%	No	%	No	%	No	%
Epithelial	Superficial	120	59.9	121	60.5	134	67.0	118	58.9
	Intermediates	53	26.3	47	23.4	39	19.7	50	24.8
	Basal cell	19	9.4	24	12.1	18	8.9	22	11.1
Calciform cells		2	1.0	2	1.2	2	1.0	2	1.1
Leukocytes	Neutrophils	1	0.5	1	0.5	1	0.6	3	1.3
	Lymphoid Cells	5	2.7	4	2.0	6	2.8	5	2.6
	Eosinophil cells	0	0.0	0	0.0	0	0.0	0	0.0
Macrophages		0	0.0	0	0.0	0	0.0	0	0.0
Erythrocytes		0	0.0	1	0.3	0	0.1	0	0.0
	TOTAL	200	100.0	200	100.0	200	100.0	200	100.0

Table 3 - Comparison of ocular conjunctiva cells between healthy cattle and horses

Cells		Cattle		Horse	
		No	%	No	%
Epithelial	Superficial	492	61.5	493	61.6
	Intermediate	164	20.5	188	23.6
	Basal cells	62	7.7	83	10.4
*Calciform cells		6	0.7	9	1.1
Leukocytes	Neutrophils	16	2.0	6	0.7
	*Lymphoid Cells	58	7.2	20	2.5
	Eosinophil cells	0	0.0	0	0.0
Macrophages		0	0.0	0	0.02
*Erythrocytes		3	0.4	1	0.1
	TOTAL	800	100.0	800	100.0

*Significant difference with ANOVA with $p < 0.05$.

Table 4- Cell maturity averages in the ocular conjunctiva of healthy cattle and horses (Shorr staining)

Cell type	Cattle Right eye Mean \pm SD	Left eye Mean \pm SD	Horses Right eye Mean \pm SD	Left eye Mean \pm SD
Immature Cells	18.68 \pm 12.6	19.70 \pm 13.3	16.75 \pm 16.0	15.70 \pm 13.7
Mature Cells	81.32 \pm 12.6	80.30 \pm 13.3	83.25 \pm 16.0	84.30 \pm 13.7

Studies performed by Bolzan et al. (2005) in dogs, showed a percentage of superficial epithelial cells of 58% in the right eye and 57.4% in the left. The intermediate epithelial cells corresponded to values of 39.2% (right) and 40.6% (left), basal cells corresponded to 1% (right) and 0.8% (left), and calciform cells corresponded to 0.4% (right) and 0.2% (left). Neutrophils represented 1% (right) and 0.6% (left) of cells, and lymphocytes represented 0.2% of cells in both eyes. In the present study, with regard to the average proportion of cells for each eye (superior and inferior region; table 1 through 2), the cattle presented 56.28% superficial epithelial cells in the right eye and 66.73% in the left, 21.72% intermediate epithelial cells in the right eye and 19.30% in the left, 9.95% basal/parabasal cells in the right eye and 5.45% in the left and 0.8% calciform cells in the right and 0.62% in the left. Neutrophils corresponded to 1.73% in the right eye and 2.32% in the left, and lymphocytes corresponded to 9% in the right and 5.4% in the left. However, in horses, the superficial epithelial cells corresponded to 60.22% in the right eye and 62.93% in the left, intermediate cells corresponded to 24.88% in the right eye and 22.27% in the left, basal/parabasal cells corresponded to 10.75% in the right eye and 10.03% in the left, and calciform cells corresponded to 1.10% in the right eye and 1.05% in the left. Neutrophils represented 0.53% in the right eye and 0.95% in the left, and lymphocytes corresponded to 2.33% in the right eye and 2.72% in the left. A comparison of the results of Bolzan et al. (2005) with the present work indicates a difference between the basal epithelial cells of dogs compared with horses and cattle.

These differences can be explained by considering the different species and the environments in which these studied animals live, which can influence the types and quantities of the cells found, especially inflammatory and superficial epithelial cells, due to the occurrence of constant trauma and the presence of foreign bodies. Furthermore, there may be a discrepancy in the method of

collection, especially regarding the pressure exerted by the finger onto the conjunctival surface, which is not as exfoliative as other methods.

Through the technique of Shorr staining, which indicates cellular maturity, mature cells were observed to be more prevalent on the ocular surface than immature cells. Among the cattle, mature cells accounted for 80.81%, with only 19.19% being immature, and a similar result was found in horses, in which 83.78% of the cells were mature and 16.23% were immature. This high percentage of mature cells may be due to the large quantity of epithelial cells, especially superficial and keratinized cells.

The statistical analyses allowed us to differentiate between the numbers of calciform cells, erythrocytes, and lymphocytes between species and between individual animals. The differences may be due to physiological and histological aspects of each species. Specifically, as the cattle were less docile, they did not permit adequate manipulation of the face (eyeball region), and consequently, a lower pressure was exerted on the filter paper at the moment of collection. Likewise, the differences in the cell numbers between animals can be influenced by the age variability in the sampled animals. However, further studies are required with larger samples to obtain higher reliability of the results and to determine whether these differences are indeed significant.

CONCLUSION

According to the results obtained, the authors conclude that impression cytology of the bulbar conjunctiva provides an adequate quantity of cells for counting and classification. This method provides good preservation of the cellular details and morphology, and it is atraumatic and well accepted by both cattle and horses.

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