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Domestic bovines as potential environmental bioindicators: analysis of oral epithelium and application in the micronucleus assay

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Abstract
Domestic cattle (*Bos Taurus*) could be used as bioindicators of the quality of agroecosystems, with the possibility of alerting through cellular biomarkers about possible adverse effects of drugs administered in them or toxic contaminants in the surrounding environment. The micronucleus buccal Micronucleus Cytome (BMCyt) assay is used in human populations for this purpose. The aim of this study was to perform the structural characterization of the epithelium in the anatomical site proposed for performing the oral MN-cyt buccal assay in this species and to describe the types and frequencies of cells with nuclear abnormalities (NA) of the bovine oral lining epithelium. Exfoliative cytology of the buccal labial epithelium of twelve castrated males was performed and 1,000 cells per animal were analyzed. The frequencies of basal and differentiated cells with NAs were established. The most frequently observed cell types and NAs were: karyolytic, condensed chromatin, karyorrhexis, pyknotic, kidney-shaped, notched nuclei, binucleated, micronucleated and buds. Four grades of progression were described in nuclei with karyorrhexis. A keratinized flat stratified epithelium of 866.67±75.44 µm thick (Mean ± SD) was evidenced and the characteristics of the cells of the strata *germinativum, granulosum, spinosum* and *corneum* are delineated. In addition to being keratinized, the bovine epithelium is three to five times thicker than that recorded in humans due to more differentiated cells. In the prospective use of BMCyt assay in bovines, indicators of cell death should not be considered as a result of genotoxic effects that induce apoptosis, as occurs in humans; the rest of the NAs could be used as biomarkers.

Key words: agroecosystem, biomarker, exfoliated cells, histology, lining tissue, cattle.

Los bovinos domésticos como potenciales bioindicadores ambientales: análisis del epitelio oral y aplicación en el ensayo de micronúcleos

Resumen. Los bovinos domésticos (*Bos Taurus*) podrían utilizarse como bioindicadores de la calidad de los agroecosistemas, con la posibilidad de alertar mediante biomarcadores celulares sobre posibles efectos adversos de fármacos administrados en ellos o de contaminantes tóxicos en el ambiente circundante. El ensayo de micronúcleos citoma bucal (MN-cit bucal) se utiliza en poblaciones humanas con este fin. El objetivo de este estudio fue realizar la caracterización estructural del epitelio en el sitio anatómico propuesto para la realización del test MN-cit bucal en esta especie y describir los tipos y frecuencias de células con anormalidades nucleares (AN) del epitelio bucal bovino. Se realizó citología exfoliativa del epitelio bucal labial de doce machos castrados y se analizaron 1.000 células por animal, estableciéndose frecuencia de células basales y diferenciadas con AN. Los tipos celulares y AN observados con mayor frecuencia fueron: cariolíticas, cromatina condensada, cariorrexis, picnóticos, en forma de riñón, núcleos

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con muescas, binucleados, micronucleados y brotes. Se describieron cuatro grados de progresión en núcleos con cariorrexis. Se evidenció un epitelio estratificado plano queratinizado de 866,67 ± 75,44 µm de espesor (media ± DE) y se delinean las características de las células de los estratos *germinativum, granulosum, spinosum* y *corneum*. Además de estar queratinizado, el epitelio bovino es entre tres y cinco veces más grueso que el registrado en humanos debido a un número mayor de células diferenciadas. En el uso prospectivo de MN-cit bucal en bovinos, los indicadores de muerte celular no deben considerarse como resultado de efectos genotóxicos que inducen apoptosis, como ocurre en humanos; el resto de las AN si pueden ser utilizados como biomarcadores.

Palabras clave: ecosistema agropecuario, biomarcador, células exfoliadas, histología, tejido de revestimiento, ganado.

INTRODUCTION

The analysis of the morphology of the superficial cells of the buccal mucosa, along with the frequency of presentation of each cell type, is used to study cell proliferation, differentiation, genetic damage, and cell death. The layers of the stratified, flat buccal epithelium include the basal or germinative layer, as well as other layers that demonstrate a higher degree of differentiation, such as the *granulosum, spinosum*, and *corneum* layers (Thomas et al. 2009, Bolognesi et al. 2013).

In human populations, the analysis of exfoliated cells from the buccal mucosa is employed in epidemiological studies, known as the buccal Micronucleus Cytome (BMCyt) assay. This assay assesses micronuclei (MN), which serve as indicators of genetic damage. MN are small fragments of chromosomes or whole chromosomes that are excluded from the main nucleus. Other nuclear abnormalities (NAs) examined include nuclear buds, suggestive of gene amplification; cells with two main nuclei due to cytokinesis failures; nucleoplasmic bridges caused by dicentric chromosomes; cells displaying morphological characteristics of cell death processes, such as condensed nuclear chromatin, karyorrhexis, pyknosis, and karyolysis (Benedetti et al. 2013, Bolognesi et al. 2013).

The BMCyt assay is applied in human biomonitoring research related to various factors, including nutrition, lifestyle habits, tobacco and alcohol consumption, risk of accelerated aging, neurodegenerative diseases, screening for precancerous and oral cancer conditions, and assessing the prognosis of certain therapies, exposure to genotoxic and cytotoxic agents, as well as exposure to environmental pollutants (such as pesticides and volatile organic compounds) (Thomas et al. 2009, Bolognesi et al. 2013, Sommer et al. 2020).

When the BMCyt assay is implemented in animal populations, these animals are considered biological indicators or sentinels of environmental quality, as they can provide early warnings about environmental health hazards and can warn of toxic effects on the health of people living in that ecosystem. If these animals are part of the agrifood production chain, such studies can also alert us to the presence of contaminants that may affect the final products destined for human consumption (Frazzoli et al. 2015).

Domestic cattle are good sentinels for environmental biomonitoring because they have limited geographical mobility and stay in a single site for long periods of time, which subjects them to xenobiotics exposure, similar to the people with whom they share the environment; they can accumulate contaminants in their bodies; and they integrate populations that are ordered and managed, so that information about blood parameters, health status, reproduction, among others, is available (Frazzoli et al. 2015). This species has been studied as a sentinel of the presence of a wide range of contaminants in soil, water, air and also contaminants present in food production, such as microplastics, heavy metals, persistent organic compounds, insecticides, among others (Van Dijk et al. 2014, Amadi et al. 2020, Ferré and Gorla 2023, Prata and Dias-Pereira 2023).

Cattle have been used as sentinels to assess the quality of agricultural environments by analyzing genetic damage in their blood cells. Researchers have evaluated the frequencies of binucleated lymphocytes with micronuclei (in the Cytokinesis Block Micronucleus Assay) and other NAs in animals exposed to agents such as neonicotinoids, triazoles, pesticides resulting from agricultural practices, dietary habits, and consumption of contaminated water (Michalová et al. 2020). Additionally, studies have examined genetic damage in cattle exposed to cypermethrin and chlorpyrifos (Ferré et al. 2020), chromosomal abnormalities in bovines residing in arsenic-contaminated regions (Shekhar et al. 2014), in proximity to dioxin-emitting factories (Di Meo et al. 2011), and micronuclei formation in cattle with access to wastewater (Lee et al. 2007). The evaluation of micronuclei in bovine erythrocytes has also been associated with various environmental pressures and natural selection (Glazko et al. 2012).

Biomonitoring studies on domestic bovine exfoliated epithelial cells, despite the advantages reported for the BMCyt assay in humans, have not been identified. The feeding characteristics and chewing habits of cattle determine the morphology of the lining epithelia of the buccal cavity, reported as flat stratified keratinized around lipstick area (Bacha and Bacha 2001). To implement the BMCyt assay in this species, it is necessary to confirm the architecture and tissue organization of the buccal epithelium from the exact anatomical area where the exfoliative cytology scraping is performed. With histology, the constituent cellular strata are identified, the thickness of each one of them and the morphological characteristics of the cells that progress in cellular differentiation to corneocytes. Once the histological characteristics of the buccal epithelium have been clarified, and after performing the exfoliative cytology required by the BMCyt assay, it will be possible to identify cell types and NAs that are

markers of genetic damage. The aim of the study was to characterize the histological features of the inner lining epithelium of the upper lip in domestic bovines and to determine the types and frequencies of abnormal nuclei presentations in exfoliated cells from the same anatomical area.

MATERIALS AND METHODS

Cytology analysis of the buccal lip epithelium. An exfoliative cytology of the buccal lip epithelium, an area where there are no papillae, of twelve castrated male Aberdeen angus and crossbred animals was performed. The animals from 15 to 18 months of age and 355.80 ± 25.75 kg live weight (mean ± SD) were from Lavalle, Mendoza, Argentina (621 masl). The animals inhabited a productive system in which no sources of anthropogenic pollutants were visually identified within a 10 km radius. This ecosystem is distinguished by its desert/arid climate, with a predominance of uncovered or native soil cover; and the main economic-productive activities are agriculture and extensive livestock farming. The animals were fed with balanced feed and supplemented with split corn and alfalfa. Prior to the study, the animals were clinically inspected by a visual observation of mucous membranes, as well as by physiological parameters such as confirmation of their temperature, heart rate and respiratory rate. Animals were selected if they presented good body condition, free of lesions, showed no signs of disease, and exhibited normal behaviors typical of the species. By means of anamnesis with the person in charge of the production system, it was confirmed that the animals had not received any medication for the three months prior to the time of the study. The design of the trial was approved by the Institutional Committee for the Care of Laboratory Animals, Experimentation and Teaching (CICUALID) of the University Juan Agustín Maza (number 79).

The Thomas et al. (2009) protocol was implemented with modifications made according to Ferré et al. (2018). Cell Samples were obtained with mini plastic spatula by scraping, 2 to 3 times, the inner lining epithelium of the bovine upper lip that had been previously rinsed with water, and then spread on slides. The slides were fixed with acetic acid: methanol (Baker®) (3:1) for 10 min, stained with 10% Giemsa (Merck®) and then placed in distilled water for 10 min. One thousand epithelial cells per animal were analyzed in a Nikon® optical microscope at 1000X. A morphological description was made and the relative values of basal and differentiated cells with different nuclear forms were established and categorized as normal nuclei, with condensed chromatin, with fragmented nucleus, without nucleus, with micronucleus, pyknosis, with nuclear buds, presence of two nuclei or binucleated, and other NAs. Slides were coded and microscopic observations were performed blind by two operators, each one analyzing half of the total cells. An images gallery was made by microphotographies.

Histological description of bovine buccal lip epithelium. Histological analysis of bovine inner lining epithelium was performed on one sample from the upper lip of an Aberdeen Angus crossbred steer immediately after slaughter. The sample was obtained from a private slaughterhouse outside the University, authorized by the competent health authority. The anatomical sampling site was exactly the same as the one where scraping is performed on live animals to obtain exfoliated cells for analysis in the BMCyt assay (Figure 1). One segment of 2 x 3 x 1 cm was obtained. The sample was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, followed by dehydration in increasing concentrations of ethyl alcohol, clarification in xylol and impregnation in paraffin. Four -six µm thick sections were cut with a Reichert sliding microtome and stained with hematoxylin-eosin (Díaz 2010). For microscopic evaluation a digital image processing Nikon Eclipse E200® microscope with 391CU 3.2M CCD camera was used. Using Micrometrics® S. E. Premium and PhotoScape v3.5 imaging software, the thickness of the different cellular layers of the epithelium was calculated, measured at three random points in each layer, from which mean values were obtained. Distances were calibrated for the 100x objective lens using image analysis software and a micrometer standard bar.

Figure 1. Photograph of the area where the tissue section was obtained to perform the histopathological study of the domestic bovine oral epithelium. Section (2 x 3 x 1 cm) of the upper lip from a castrated male bovine was obtained post-slaughter. The arrow indicates the exact part that was sectioned for histological analysis. This same anatomical site is the one used to scrape with mini spatulas and obtain exfoliated cells for the BMCyt assay.

Statistical analysis. The GraphPad Prim 6.0® program was used for statistical analysis. It was determined whether the frequencies of each cell type and NA followed a normal distribution according to the Shapiro-Wilk test, and the mean values of each variable studied were obtained. Pearson's test was implemented to evaluate the correlation between basal cells and non-nucleated cells.

RESULTS

Exfoliative cytology revealed basal cells, intermediate and superficial differentiated cells typical of keratinized stratified flat epithelium (Figure 2.1). The basal cells are smaller than the differentiated cells, although they have a large rounded nucleus that occupies a large part of the basophilic cytoplasm. Intermediate differentiated cells have a larger cytoplasm to nucleus ratio, low basophilia and polyhedral morphology. Towards the surface, differentiated cells are visualized with acidophilic cytoplasm and others with basophilic granules and mostly without nuclei. Differentiated cells with diverse morphological nuclei characteristics are observed.

The frequencies of basal and differentiated exfoliated cells with different NA found are presented in Table 1. The correlation between the frequencies of basal cells and without nuclei cells was r = -0.19 (p = 0.55). The most frequently observed cell types and NAs were: kariolytics, condensed chromatin, karyorrhexis, pyknotic, kidney shaped, notched, binucleated cells and in equal proportion micronucleated and nuclear buds. No cells with nucleoplasmic bridges were found. Cells containing nuclei with condensed chromatin have presented varying degrees of nuclear condensation due to the appearance of fragmented or karyorrhexis nuclei. A classification of karyorrhexis cells was specified, based on the characteristics of coloration, texture and degree of nuclear fragmentation. (Figure 2.2).

Table 1: Frequencies of exfoliated cell types and nuclear morphologies from the buccal cavity of twelve healthy steers.

Cellular types and nuclear abnormalities	Number/ 1000 cells (mean ± s.e.)
Basal cells	24.17 ± 3.55
Differentiated cells	
Normal nucleus	350.50 ± 37.93
Micronucleated	0.08 ± 0.08
Nuclear buds	0.08 ± 0.08
Nucleoplasmic bridge	0.00 ± 0.00
Kidney shaped	5.00 ± 0.72
Notched	2.16 ± 0.34
Binucleated	0.16 ± 0.11
Condensed chromatin	112.60 ± 17.20
Karyorrhexis	18.83 ± 2.99
Karyolitic	471.80 ± 42.10

s.e. standard error.

The histological study of the internal lining epithelium of the domestic bovine upper lip showed a keratinized stratified flat epithelium 866.67 ± 75.44 µm thick (Mean ± SD) (Figure 3). In the basal layer (*stratum germinativum*), a single layer of cells, mainly keratinocytes, with large cubic or cylindrical and basophilic nuclei, was observed. The cells have little cytoplasm, elongated hexagonal shape and in some cases presence of brown melanin pigment granules. Melanocytes with cytoplasm large, oval nuclei surrounded by abundant brown granulations were observed among the keratinocytes. Other "clear cells" were observed with several nucleoli, lobulated and/or "fusiform" nuclei, which also showed a clear perinuclear halo (Figure 4.1).

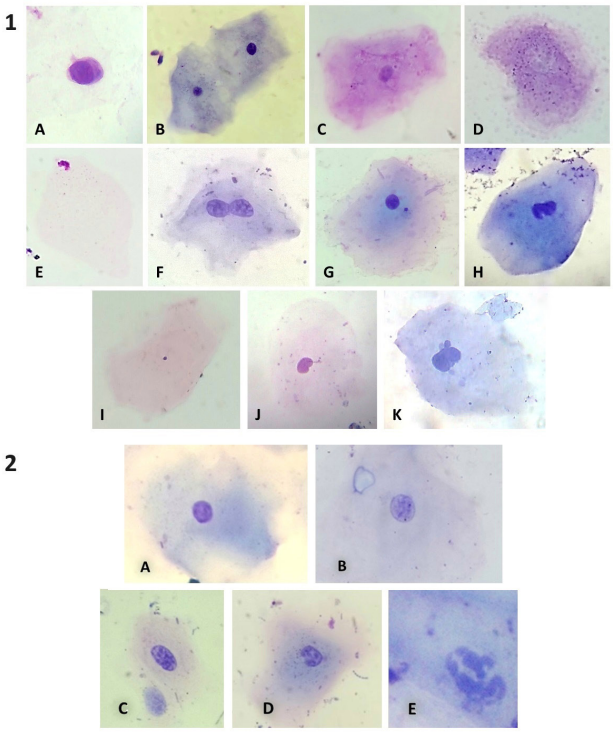

Figure 2. Photo gallery of the different types of cells found in cattle buccal epithelium. Exfoliated cells from the inner lining epithelium of the bovine upper lip, stained with Giemsa, 1000X. **1.** Normal cells, cytokinesis defects, cells indicating genetic damage, and cell death. Giemsa 10% stain. A- Basal cell, B- Intermediate differentiated cell with normal nucleus, C- Superficial differentiated cell with normal nucleus, D- Cell without nucleus and cytoplasm with basophilic keratohyalin granules, E- Cell without nucleus, F- Binucleated, G- Micronucleated cell, H- Notched, I- Pyknotic nucleus, J- Kidney-shaped nucleus, K- Nuclear bud. **2.** Cells with different degrees of nuclear condensation. Giemsa 10% stain. A-Grade 1, cells with mild condensed chromatin, a heterogeneously colored nucleus is observed. B- Grade 2, cells with mild to moderate condensed chromatin, in which nuclei with moderate-sized not stained gaps are observed. C- Grade 3, cells with moderate to intense condensed chromatin, nuclei with well-demarcated basophilic granules which pronounce small lacunae without staining. D- Grade 4, cells with intense condensed chromatin, characterized by nuclei with larger basophilic granules and large gaps without staining, giving a brindled or banded appearance.

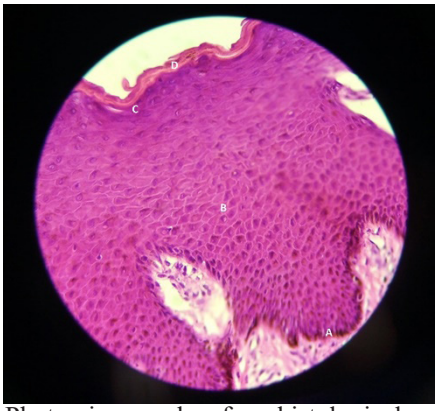

Figure 3. Photomicrograph of a histological section, domestic bovine buccal inner lining epithelium. Thickness BMCyt assay of the buccal epithelium strata from a segment

of the bovine upper lip, stained with haematoxylin-eosin, 100X. A- Basal *stratum* o *germinativum* 19.37 ± 5.07 µm, B- *spinosum* 741.40 ± 66.63 µm, C- *granulosum* 80.88 ± 1.73 µm, D- *corneum* 25.02 ± 3.03 µm. Total measurement 866.67±75.44 µm thick (mean ± SD).

The *stratum spinosum* is composed of abundant cells distributed in several layers. Two cellular morphologies are appreciated. The deeper, less differentiated (transitional or deep *stratum spinosum*), with small nucleated cells with lax chromatin and slightly basophilic cytoplasm and numerous melanin granules. The junctional boundaries between cells can be observed. Towards the surface, the cells present cytoplasm increasingly more acidophilic and more abundant in relation to the size of the nucleus. The nuclei are rounded with lax chromatin and have one or more prominent round nucleoli. The granules in the cytoplasm are lighter and less abundant (Figure 4.2). Cells with ovoid nuclei with two or three nucleoli and a clear perinuclear halo are also observed in this layer. The *stratum granulosum* consisted of two or three layers of flattened cells. Additional cells without nuclei are observed. The nuclei are colored with low intensity rounded near the *stratum spinosum,* and more flattened towards the surface. Cytoplasm without basophilia, and towards the surface, light acidophilic cytoplasm with intensely basophilic keratohyalin granules. The superficial *stratum corneum* is composed of cells without nuclei, flat and with a thick cytoplasmic membrane appearance. In this stratum there are two segments, one more superficial or disjunct, and the other deeper or lucid (Figure 4.3).

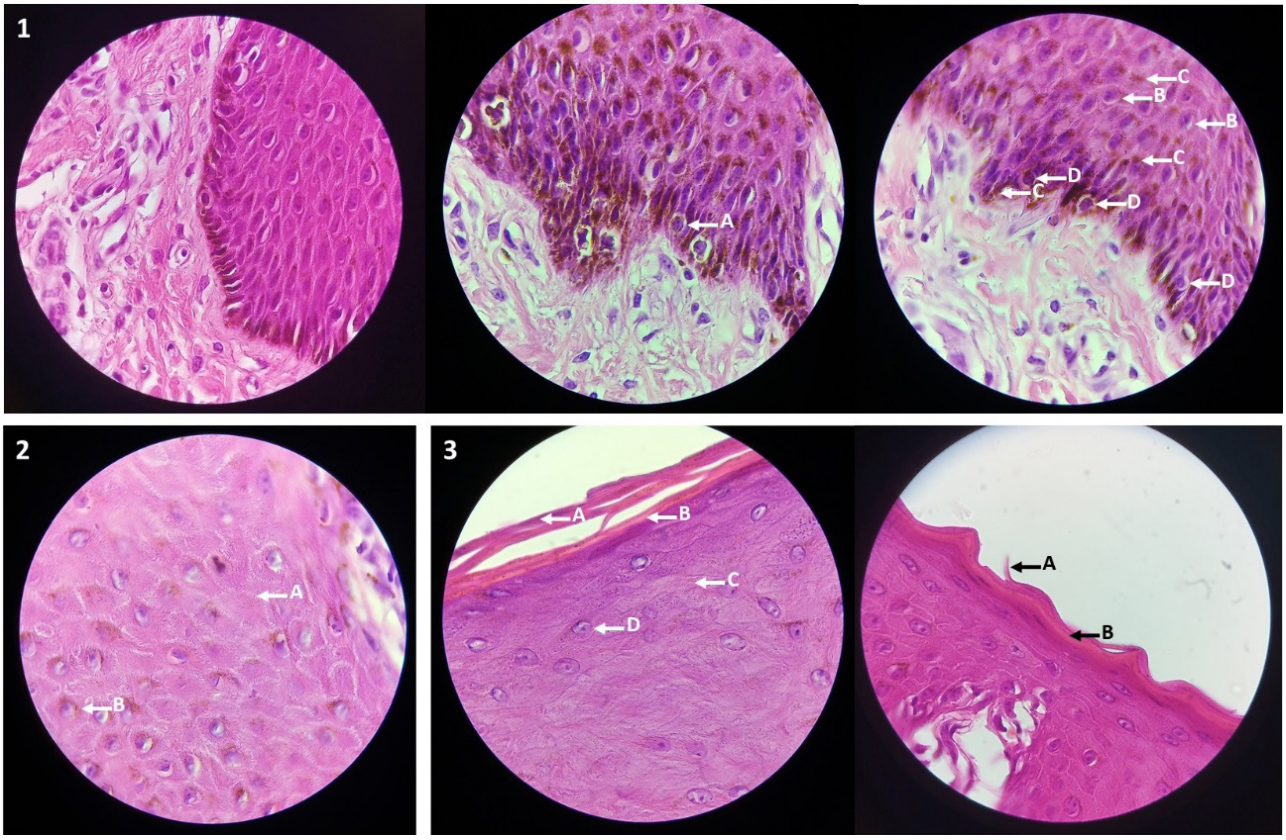

Figure 4. Histological photomicrograph, tissue layers of the bovine buccal inner lining epithelium. Routine hematoxylin and eosin-stained section, 1000X. **1**. *Stratum germinativum*. A- Melanocyte with nucleus surrounded by melanin granules, B- Clear cells, C- Brownish melanin granules yielding to keratinocytes and distributed in various cell layers, D- Basal cells with intense basophilic staining. **2**. *Stratum spinosum.* A- Intercellular bridges, B- Keratinocyte with acidophilic cytoplasm and nucleus surrounded by melanin. **3**. *Stratum granulosum* and *corneum*. A- Disjunct, B- Lucid, C- Anucleate keratinocyte, D- Keratinocyte with identifiable nucleus and nucleolus, acidophilic cytoplasm with basophilic keratohyalin granules.

DISCUSSION

The morphological description of the buccal exfoliated cells of 12 healthy steers allowed us to obtain frequencies of cell types and NAs that could be of reference for future epidemiological studies. In previous studies we were able to confirm that these types of NAs were also observed in peripheral blood lymphocytes from cattle (Ferré et al. 2020). These nuclear abnormalities can occur spontaneously or can be induced by various genotoxic agents. It is essential to establish highly stringent inclusion criteria for distinguishing micronuclei (MN) and accurately documenting them as such. Giemsa-stained cell smears can sometimes exhibit morphological artifacts resembling MN, which can result from factors like excessively strong or weak staining due to the use of outdated dyes, inadequate staining times, alkaline pH of the solution, or the presence of precipitates stemming from the use of contaminated slides, among other potential causes (Cerón Madrigal 2013). Differential Feulgen DNA staining (Thomas et al. 2009) is recommended for epidemiological studies to monitor damage to the genetic material of different animal populations.

Cells with condensed chromatin, karyorrhexis, pyknotic and karyolitic cells are characteristic of the normal process of cell differentiation. When analyzed in the MN-cyt assay they provide information about the rate of cell proliferation and are indicators of cell death by apoptosis, which usually increase when cells are exposed to harmful agents (Thomas et al. 2009, Bolognesi et al. 2013). We highlight here, the characterization of different degrees of chromatin condensation in bovine epithelial cells (Figure 2.2).

NAs of exfoliated cells of the buccal epithelium, reported here for the first time for the species, have been found, with some exceptions, in other mammals. Recently, Bertolino et al. (2023) studied the types and frequencies of NAs in cells of the buccal epithelium and palate of wild grey squirrels (*Sciurus carolinensis*) from a suburban area of Italy and wild boars (*Sus scrofa*) from a protected natural park. The authors do not report frequencies of notched cells, although they include the category "broken eggs". We highlight the presence of kidney-shaped cells in these two wild species. The mechanism of formation of this type of NA is not known, and it has not been described in humans but it has also been described in our study with bovines. The BMCyt assay has also been implemented in different bat populations in Brazil. The authors discovered cells with extensive genetic damage in these animals residing in an area exposed to high levels of emissions from automobiles, as well as consumption of contaminated water and food, and in particular environments such as open-pit mines where animals are exposed to heavy metals via dermal, respiratory and oral exposure. The different populations studied show increased frequencies of micronucleated cells and other NAs according to the area studied and the type of feeding, demonstrating that the species is indicative of environmental contamination. The authors do not describe the notched and kidney shaped cell types (Benvindo- Souza et al. 2019a,b, 2023, Folador Sotero et al. 2023). Other researchers have applied the assay to cells from the buccal epithelium of canines and felines in animal shelters, revealing higher frequencies of cells with MN when compared to canines and felines with owners living in environments conducive to animal welfare. In addition to micronucleated cells, the authors present frequencies of cells with nuclear buds and a category called "nuclear rearrangement", without describing the types of NA that constitute it. However, the photographs show the image of a cell that they have called "identation", characterized by having a notch in the periphery which, in bovines we have identified as notched according to our own studies where we have observed it in avian erythrocytes (Quero et al. 2016, Santovito et al. 2022).

Because the implementation of the BMCyt assay in non-human mammalian populations is relatively recent, particularly in cattle, and there is little information on the cell types that constitute the epithelium, we propose to extend the study of exfoliative cytology of the buccal cavity in other populations of domestic cattle from different habitats without obvious environmental contamination. Then, to implement the BMCyt assay in populations with suspected genotoxic exposure. In both situations, the individuals participating in the study should be of the same breed, healthy, free of exposure to confounding factors, and without variations in the ratio between females, males and ages.

Differentiation of cells from the *stratum germinativum* to more superficial strata involve structural changes associated with lipid and protein synthesis related to the process of keratinization or cellular differentiation, and the differential expression of acidic and basic keratins. These changes are reflected in a greater or lesser affinity of the cytoplasm/nucleus to basic or acidic dyes from the Giemsa solution. The characteristic basophilic staining of basal cells is due to the large rough endoplasmic reticulum and high number of ribosomes in the cytoplasm with affinity for basophilic dyes, and to the low-density keratin filament bundles. In higher strata the keratin tonofilament bundles become increasingly dense and the cells acquire acidophilic characteristics (Figure 4). The cytoplasm of the cells of the *stratum granulosum* presents a lattice of dense keratin filaments with granules of the protein profilaggrin, which gives a basophilic coloration, commonly known as keratohyalin granules (Ross et al. 2012, Haftek and Simon 2020). Bolognesi et al. (2013) refer to these cells as intermediate differentiated cells (typical of the *stratum spinosum*, in which the cells are linked by desmosomes), and superficial differentiated cells (cells of the *stratum granulosum*) (Figure 2.1). The cornified keratinocytes or corneocytes do not present nuclei and their cytoplasmic organelles have also been autolyzed, they have a condensed appearance due to the packing of keratin located in a dense matrix; the loss of intercellular junctions or corneodesmosomes results in the desquamation of these cells. The characteristics of the "clear cells" with perinuclear light white halo found between the keratinocytes of the *stratum germinativum* may correspond to melanocytes, Merkel or Langerhans cells (Figure 4.1). These cell types cannot be distinguished by routine haematoxylin-eosin staining. The latter are present in all layers and mainly in the *stratum spinosum* (Figure 4) (Ross et al. 2012).

The cytological study of buccal epithelial samples from healthy cattle, including basal and superficial cells, showed that approximately 47% of them were cells without nuclei. Based on the histological study of the lip section, the high presence of cells without nuclei is due to the fact that the epithelium of the buccal cavity in the area where the sample was taken for the exfoliative cytology is a keratinized stratified flat epithelium. The domestic bovine epithelium is between three to five times thicker in comparison to that reported for humans (Prestin et al. 2012, Sa et al. 2016). In the upper lip area, we observed tissue with a thickness of 866.67 ± 75.44 µm, while the literature reports between 169.2 ± 37.1 µm to 294 ± 68 µm thick in the cheek area of humans. The thickness of buccal mucosal epithelium varies according to different areas. In sheep and buffalo ruminants, both the buccal lining epithelium and the palate (masticatory) epithelium are keratinized, presenting a distinction in their thickness. The buffalo palate and buccal epithelium measures 800 µm and 600 µm respectively (Sa et al. 2016), which is in agreement with our results in domestic bovine. While other authors reported a bovine palate epithelium thickness of 420 ± 20 µm (Ren et al. 2016). A grass-based diet and rumination

behaviors could promote keratinization of the epithelium and balance out the regional variations between palatal and buccal epithelium.

Some authors define the epithelium of the inner cheek epithelium of humans as non-keratinized stratified flat (Ross et al. 2012); while Thomas et al. (2009) describe a keratinized stratified flat epithelium. Despite this lack of agreement, the presence of cells without nuclei obtained from exfoliative cytology of the inner cheek is considered indicative of cell death. These cells are referred to as karyolytic cells, and populations of individuals exposed to specific contaminants exhibit elevated frequencies, therefore are studied as an excellent marker of genetic damage that promotes apoptosis due to exposure to genotoxic agents (Thomas et al. 2009, Ferré et al. 2018). This statement underscores the significance of complementing cytological studies with histological examinations, particularly in species that have not yet been characterized for this assay; because a high proportion of cells without nuclei may be normal in the process of cell differentiation or markers of cell death, condition that we have demonstrated for the domestic bovine. In this species, due to the type of keratinized stratified flat epithelium and the greater thickness of the strata with cells differentiating into corneocytes, cell types associated with cell death are abundant and represent the highest proportion in the cytology (Table 1). These cell types may not be good markers of genotoxic effect in environmental biomonitoring studies.

On the other hand, basal cell frequencies showed a wide range in the extreme values with minimum and maximum values between 12 and 51 basal cells per 1000 analyzed cells (Table 1). Basal frequencies reported for humans, are also of wide variability (Benedetti et al. 2013). Basal cells constitute the deepest layer of the epithelium, as opposed to the more superficial cells, which are cells without nuclei. In our study we observed a negative correlation, which allows us to interpret that the higher the frequency of cells without nuclei, the lower the frequency of basal cells. The way in which the samples are taken for cytological study is a factor that could influence these frequencies. It is considered desirable that sampling could be performed by a robotic system that could collect the cells uniformly by maintaining a constant tension against the internal wall of the oral cavity (Bonassi et al. 2009, Bolognesi et al. 2013).

In conclusion, in the exfoliated buccal lip epithelium from healthy cattle the predominant cells are anucleated (karyolytic), followed by other cells indicative of cell death (condensed chromatin, karyorrhexis, pyknotic), and to a lesser extent cells indicative of genetic damage. Therefore, in the potential application of the buccal cytome assay in cattle, indicators of cell death could not be considered markers of genotoxic effect, as used in humans, and it is suggested to use the following NAs: kidney-shaped nuclei, notched nuclei, micronucleated, buds and binucleated cells.

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