Chapter

Genomic Instability and Cyto-Genotoxic Damage in Animal Species

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Abstract

Genomic instability is a condition that may be associated with carcinogenesis and/or physiological disorders when genetic lesions are not repaired. Besides, wild, captive, and domesticated vertebrates are exposed to xenobiotics, leading to health disorders due to cytogenotoxicity. This chapter provides an overview of tests to assess cytogenotoxicity based on micronuclei (MNi) formation. Bone marrow micronuclei test (BmMNt), peripheral blood erythrocyte micronuclei test (PBMNt), and lymphocyte cytokinesis blocking micronuclei assay (CBMN) are discussed. The most illustrative studies of these techniques applied in different vertebrates of veterinary interest are described. The values of spontaneous basal micronuclei in captive, experimental, and farm animals (rodents, hamsters, pigs, goats, cattle, horses, fish) are summarized. In addition, a flow cytometry technique is presented to reduce the time taken to record MNi and other cellular abnormalities. Flow cytometry is helpful to analyze some indicators of genomic instability, such as cell death processes and stages (necrosis, apoptosis) and to efficiently evaluate some biomarkers of genotoxicity like MNi in BmMNt, PBMNt, and CBMN. The intention is to provide veterinary professionals with techniques to assess and interpret cytogenotoxicity biomarkers to anticipate therapeutic management in animals at risk of carcinogenesis or other degenerative diseases.

Keywords: Genomic instability, Micronuclei, MNi in erythrocytes, CBMN, Flow cytometry

1. Introduction

Genetic instability results from alterations induced by agents that severely damage DNA. The nature of the damage may be silent when it occurs in non-coding regions and therefore does not affect the cellular processes of organisms. Still, when damage occurs in key DNA segments, the biological functionality of cells, tissues, organs, and eventually organisms in a population is compromised [1]. In this sense, genotoxic and cytotoxic damage are indicators of genomic instability. Genotoxicity

involves changes in DNA structure such as aneugenic (loss of whole chromosomes) and clastogenic effects (loss of chromosome fragments); whereas cytotoxicity involves alterations in proliferation and cell cycle rate, as well as the magnitude and type of cell death (necrosis and apoptosis) [2, 3].

Various toxicological techniques can assess genotoxicity and cytotoxicity induced by physical, chemical, or biological agents. There are numerous models that can evaluate genotoxicity and cytotoxicity, ranging from biochemical and spectrophotometric tests. These assays depend on sophisticated equipment and the use of expensive reagents and consumables, compared to the set of techniques presented here, which do not require expensive equipment and are accessible to any laboratory with an optical microscope and cell staining systems. Above all, these techniques provide a deep understanding of the biological and cellular mechanisms involved in each model [4].

Assays that record the number of micronuclei (MNi) and other nuclear abnormalities are very versatile, inexpensive, and can be used in a wide variety of *in vitro* and *in vivo* models. Various techniques are based on MNi formation with applicability in the veterinary field, starting from the theoretical principle described in mouse bone marrow [5, 6]. Different techniques were also developed, such as MNi formation in mouse peripheral blood erythrocytes [7, 8] and other mammals (primates, ungulates, felines, and a wide variety of vertebrates, fish, birds, and amphibians) [9–11]. Also, MNi in lymphocytes by cytokinesis blockade (CBMN) is widely applicable in veterinary medicine because it can be developed both in cell lines and in almost any organism (humans, rodents, rabbits, fish, dogs, primates, etc.), whose entire blood volume allows extraction of at least 0.5 mL of whole venous blood [4, 12].

Despite these advantages, techniques based on MNi formation require manual counting with light or fluorescence microscopy. Therefore, reviewer training is crucial due to the time expenditure (2 to 4 hours per slide) and accuracy in distinguishing MNi and other cellular abnormalities [13, 14]. Besides, flow cytometry offers an alternative to reduce the time spent on the microscope by standardizing observations. Initially, this technique required lysing the cytoplasm to release MNi, and thus facilitate their identification [15, 16]. However, this prevents the observation of nuclear buds (NBUDs) nucleoplasmic bridges (NPBs), which are observed in binucleated cells [17]. Subsequently, flow cytometry was improved with image flow cytometry (IFC) techniques that efficiently and automatically record mono-, bi-, and polynucleated cells with and without MNi, NBUDs, and NPBs, which is possible by combining the image flow cytometry technique with the machine learning approach [18].

2. Basis of the bone marrow micronucleus test (BmMNt)

In 1973, the bone marrow micronucleus test (BmMNt) was reported as being a more effective in determining chromosomal damage than the metaphase scoring method used at that time. Nevertheless, limitations of this method included the use of high concentrations of metaphase cells to quantify significant differences, in addition to the animal sacrifice requirement [19]. Then, in 1975 W. Schmid reported the principles of BmMNt, describing that MNi result from a malfunction in the cell division process, mainly in two different ways [5]:

Case 1: Acentric or fragment chromosomes do not migrate to the spindle poles in anaphase stage of cell division. Then MNi can be seen in the daughter cells.

Case 2: After one or more mitoses of exposed cells to mutagen agents, if the mitotic spindle is damaged, the nucleus of daughter cells could contain many MNi of a larger size than those produced in case 1.

Schmid manuscript also describes erythrocytes derived from the bone marrow as the best cell type to perform the assay since distinguishing between immature erythrocytes (polychromatic) and mature (normochromic) erythrocytes is possible, considering immature erythrocytes remain in circulation for 24 to 48 hours, while mature erythrocytes remain for about 30 days [5].

2.1 Mammalian bone marrow erythrocyte micronucleus test (MEMT)

Chromosomal damage, genome instability, and cancer risk assessment are the main objectives of bone marrow erythrocyte micronucleus assay (BmMNt) [20]. Its robustness lies in the fact that it determines in a simple and relatively fast way the clastogenicity or aneugenicity of chemicals [21]. The mammalian erythrocyte micronucleus test (MEMT) has been widely reported and reviewed by different research groups and government agencies. However, since its publication, more than 30 years of evidence was compiled to standardize the procedure to ensure its applicability. In addition, MEMT has been compared with other mutagenicity assays, which include the mutation in mouse lymphoma cells L5178Y, *Salmonella typhimurium*, sister chromatid exchanges, and chromosomal aberrations in Chinese hamster ovary cells [22].

The number of cells required for an appropriate genotoxicity analysis was defined by the statistical analyses of all the techniques used to evaluate MNi formation, including the MEMT technique [23, 24]. On the other hand, the preferred species for this technique are mice, rats, and Chinese hamsters. Therefore, in vivo testing is usually performed on rodent's bone marrow erythrocytes. However, other mammals, in which the spleen does not filter efficiently micronucleated erythrocytes, are accepted if stain accuracy is evaluated [24–26].

3. Potential uses of peripheral blood micronucleus test (PBMNE)

The peripheral blood erythrocyte micronucleus test (PBMNE) is used for ecotoxicological studies, monitoring of health effects from anthropogenic contamination, and genotoxic evaluation of pharmacological therapy administered in patients with chronic diseases [1]. Regarding the experimental procedure, mice are the most commonly used animals [2]. However, there are more animal models such as the rat and hamster [3] and others not as common like primates [4], birds [5], reptiles [6, 7], amphibians [8, 9], embryos [10], and fish [11]. Peripheral blood is the most versatile tissue for genotoxic and cytotoxic analysis. It is possible to use polychromatic and normochromatic erythrocyte conditions to explain the effects of myelosuppression and DNA damage [12]. Like all diagnostic tests, it has its limitations, which must be considered to avoid false negatives. One of them is that it does not detect substances that do not produce fractures or anaphase lags (aberrations that do not imply the occurrence of acentric fragments, for example, translocations and inversions); it is also not valuable for cells exhibiting a low rate of cell division or when organ-specific or species-specific carcinogens are tested [13].

Therefore, if all industrialization processes have the potential to generate large amounts of genotoxic substances, it is necessary to implement new models, such as plants or animals, to evaluate whether a particular substance or agent is harmful in the short/long term due to its mutagenic, clastogenic or aneuploidogenic, and teratogenic properties. Furthermore, to define toxic doses with greater precision, studies in several bioindicator models and not only in one must be carried out [14]. For the selection of any organism (plant or animal) as a toxic biomonitor, its cost, convenience, sensitivity, and possible extrapolation to other organisms or situations must be justified [15].

3.1 Peripheral red blood micronuclei assay

Peripheral blood was selected as a non-invasive sample to perform the MNi assay considering the invasive procedure implicated in a bone marrow sample. MNi are characterized by having a round or almond shape, with a diameter that varies from 1/20 to 1/5 (0.4 to 1.6 μ) of the average erythrocyte size (6 to 8 μ in diameter). William Henry Howell and Justin Marie Jolly identified MNi in erythrocyte precursors at the end of the 19th century and described them as remnants of the nucleus of circulating erythrocytes. Therefore, they are called Howell-Jolly bodies [16]. Subsequently, Dawson described MNi in the bone marrow of patients with several pathologies, including deficiency of cobalamin and folates; thereafter, MNi were described in lymphocytes [17].

Young or polychromatic erythrocytes (EPC) lose ribosomes within 24 hours after enucleation but retain MNi; later, they reach maturity and are transformed into normochromatic erythrocytes (ENC). These are stained blue-gray with the Giemsa stain or red with acridine orange, facilitating their identification when they are counted in tests of short exposure periods [1, 18]. Under certain circumstances, micronucleated erythrocyte (MND) values are often altered. Regardless of the tissue that is used in the MNi test, the data obtained are highly informative since it is a diagnostic tool to detect the loss of genetic material when these structures are identified in the cytoplasm of cellular compartment of the analyzed sample [19, 20].

3.2 Peripheral blood and mononuclear phagocytic system MNi test

The mononuclear phagocytic system (MPS), formerly called the reticuloendothelial system, is responsible for eliminating old or altered red blood cells, including micronucleated cells. In addition, the MPS system plays a key role in regulating innate immunity and it is constituted by dendritic cells, macrophages, and monocytes. The spleen, which is rich in macrophages, is the most sensitive detector for any red blood cell abnormality. By filtering the blood, the spleen eliminates foreign particles through phagocytic cells and destroys old erythrocytes or their fragments caused by structural changes that reduce their flexibility, making it difficult to pass through the microcirculation, undergoing cell lysis and splenic clearance [14, 21].

In mammals, two types of spleen are described, “defensive” and “storage.” The former is smaller in size, has less muscle, but is abundant in lymphatic tissue and sinusoids; the latter, is larger, scarce in sinusoids, rich in the red pulp, and stores a more significant amount of blood [21]. Nevertheless, some species have a “defensive” spleen, which eliminates the abnormal erythrocytes in their entirety, making it impossible to observe MNi in peripheral blood. On the other hand, species with “storage” spleen are deficient in their phagocytic function and allow MNi to be observed at any time during the life of the species, as in the case of mice [14].

The number of MNi in peripheral blood is practically null in humans [22]. However, they can be observed in impaired splenic function secondary to pathologies that directly affect it, for example, when patients have been splenectomized or were born prematurely. Since it is ethically not allowed to carry out biomonitoring programs in humans, these type of bioassays provide the opportunity to test genotoxic agents [14, 17, 22].

The organism’s age influences the variability in the number of MND [23] demonstrated when analyzing their frequency in splenectomized patients since adults showed a higher frequency than children [24, 25]. Similar results are described in rodent spermatids, where old mice and hamsters have more MNi than young ones, probably because genetic damage continually accumulates throughout the organism’s life [25, 26]. Some organisms present a higher frequency of MNi in juvenile

Group	Species	E / Ex	Treatment (Doses) /Analyzed zone	Frequency	Total Erythrocytes	Ref
Primates	Capuchin monkeys *Cebus capucinus*	E	Captivity	20.5 ± 2.0	10, 000	[4, 14]
	Common marmoset *Callithrix jacchus*	Ex	- Water 0.2 ml - Methotrexate 2.5 mg/kg - Cytosine arabinoside 3 mg/kg	8.0 ± 3.3 22.0 ± 5.7 31.2 ± 10.3	10, 000	
Carnivores	Cougar *Puma concolor*	E	Captivity	18.5 ± 0.7	10, 000	[14]
	Tiger *Panthera tigris*	E	Captivity	20.5 ± 2.9	10, 000	
	Lion *Panthera leo*	E	Captivity	0.6 ± 0.1	10, 000	[28]
Rodents	Guinea pig *Cavia porcellus*	E	Captivity	0.3 ± 3.0	10, 000	[28]
	Yellow-necked mouse *Apodemus flavicollis*	E	Captivity	0.2 ± 0.01	2, 000	[3]
	Common vole *Microtus arvalis*	E	Captivity	0.03 ± 0.01	2, 000	
	Mouse *Mus macedonicus*	E	Captivity	0.02 ± 0.01	2, 000	
	C57BL / 6	E	Captivity	0.3 ± 0.1	2, 000	[29]
Chiropters Marines	Bat *Pteronotus mexicanus*	E	Captivity	0.06 ± 0.04	1, 000	[30]
	Common Bottlenose dolphin *Tursiops truncatus*	E	Captivity	24.3 ± 6.1	10, 000	[31]

Group	Species	E / Ex	Treatment (Doses) /Analyzed zone	Frequency	Total Erythrocytes	Ref
Ungulates	Beef cattle *Bos taurus*	E	Captivity	0.08 ± 0.2	3, 000	[32]
	Sheep *Ovis aries*	E	Captivity	1.0 ± 0.7	3, 000	[32]
	Horse *Equus ferus caballus*	E	Captivity	0.2 ± 0.3	3, 000	[32]
Birds	Helmeted manakin *Antilophia galeata*	E	Captivity	1.1 ± 1.2	10, 000	[5]
	Golden-crowned warbler *Basileuterus culicivorus*	E	Captivity	2.0 ± 1.2	10, 000	
	Gray-headed tanager *Eucometis penicillata*	E	Captivity	2.4 ± 1.6	10, 000	
	Flavescent warbler *Myiothlypis flaveola*	E	Captivity	2.0 ± 1.8	10, 000	
	Orange-fronted Parakeetaratinga canicularis	Ex	Mitomycin C 2 mg/kg	6.0 ± 3.3	10, 000	[33]
Reptiles	Lizard *Tupinambis merianae*	E	Captivity	1.0 ± 0.2	1, 000	[6]
	Caiman *Caiman latirostris*	E	Captivity	1.1 ± 0.7	1, 000	[7]

Group	Species	E / Ex	Treatment (Doses) /Analyzed zone	Frequency	Total Erythrocytes	Ref
Amphibians	American bullfrog *Lithobates catesbeianus*	E	Captivity	3.6 ± 2.8	1, 000	[34]
		Ex	Radiation 3.3 Gy	7.3 ± 3.1	1, 000	
	Frog *Physalaemus cuvieri*	E	Emas National Park	0.2 ± 0.6	1, 000	[9]
	Lesser Treefrog *Dendropsophus minutus*	E		0.2 ± 0.4	1, 000	
	Mole salamanders *Ambystoma sp.*	Ex	Cyclophosphamide 75.0 mg	6.4 ± 2	2, 000	[8]
Fishes	Brown trout *Salmo trutta*	E	Gafo River	2.4 ± 1.9	1, 000 (renal erythrocytes)	[11]
		E	Trubia River	4.1 ± 1.3		
	Common carp *Cyprinus carpio*	E	Trasimeno River	0.5 ± 0.2	25, 000	[35]
		Ex	CH_3COO_2H NaClO ClO_2	0.8 ± 0.3 2.5 ± 0.5 1.7 ± 0.4	25, 000	
	Fish *Tilapia, sp.*	E	Xochimilco River	7.4 ± 5.7	10, 000	[36]
	Astyanax bimaculatus	Ex	Cyclophosphamide 16 mg/ kg	2.0 ± 0.7	1, 000	[37]
		Ex	Vinblastine Sulfate 8 mg/kg	1.2 ± 0.6	24, 000	

E: environmental, Ex: experimental

Table 1.
Examples of experimental species used in the MNi erythrocyte test as environmental biomonitors.

stages due to the immaturity of their nuclear phagocytic system; upon reaching adulthood, their system becomes efficient and prevents the visualization of MNi [14, 25].

3.3 Selection of a suitable peripheral red blood micronucleogenicity bioindicator

To properly select a biomonitor for the MNi test in peripheral blood, at least six MNi in a total count of 10,000 erythrocytes should be identified [25]. The analyzed tissue must meet the following requirements: be in constant division, have abundant quantified cells, sufficient cytoplasm-nucleus relationship to identify MNi clearly, and a regular shape of the nucleus without lobes must be present to facilitate their observation [27]. This assay has been applied in a broad diversity of organisms to take advantage of the available resources in the environment (**Table 1**). The investigations carried out by Dr. Zúñiga's group concluded that the organisms with the best potential are felines, the capuchin monkey, and the atolero parakeet, among others [4, 14, 19, 38, 39].

4. The cytokinesis blocking micronucleus assay (CBMN)

The cytokinesis blocking micronucleus assay in lymphocytes (CBMN) was developed by a Ph.D. student more than 30 years ago [40], who anecdotally relates that while reviewing a biochemistry textbook [41]. He noticed that cytochalasin-B had the ability to block the action of actin *in vitro* cultures of human lymphocytes and thus obtain binucleated cells capable of recording clastogenic or aneugenic events resulting from exposure to xenobiotics. This biochemical principle is a fundamental aspect, enabling binucleated cells to remain in telophase (**Figure 1**). CBMN ensures that binucleated cell have undergone a single cell duplication in culture 72 hours after its initiation (**Figure 1**), making it possible to record cytotoxic

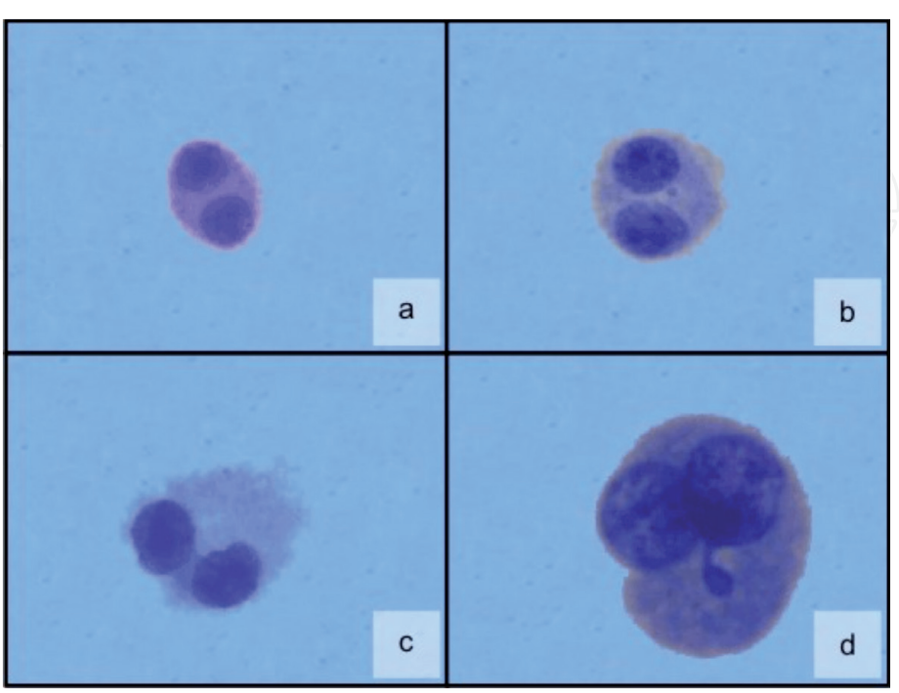

Figure 1.
Human lymphocyte binucleated cells: a) Binucleated normal cell (BNC), b) Binucleated cell with micronucleus (MNi), c) Binucleated cell with nucleoplasmic bridge (NPBs), d) Binucleated cell with nuclear bud (NBUDs).

or genotoxic events before blocking cytokinesis based on the following biomarkers: micronuclei (MNi), nuclear buds (NBUDs), nucleoplasmic bridges (NPBs), as well as mononucleated, binucleated, trinucleated and tetranucleated cells, which are used to calculate the cell duplication index (NDI); also the number of cells in necrosis and apoptosis can be recorded to perform a complete analysis of genomic instability [42–44].

4.1 General procedure for the cytokinesis-block micronucleus (CBMN) assay in lymphocytes

The experimental procedure begins by extracting whole venous blood in heparinized tubes. According to the designed experiment, cultures are prepared with 6.3 mL of RPM1–1640 medium supplemented with non-essential amino acids, 0.2 mL of phytohemagglutinin, and 0.5 mL of whole venous blood incubated for 44 hours at 37°C. After this time, between 3 and 6 μg/mL of cytochalasin-B is added to avoid the division of the cytoplasm (blocking cytokinesis), and incubation is resumed until 72 hours are completed (**Figure 2**).

Once the 72-hour incubation period is finished, cells must be fixed with Clarke's solution and washed 3 to 5 times until a clear cell button is obtained. If necessary, impurities are removed with a trypsin solution. The cell button is transferred on slides and stained with eosin and methylene blue to record nuclear abnormalities (**Figure 1**) in a total of 1000 binucleated cells and count in 500 cells those mononucleated, binucleated, trinucleated, and tetranucleated cells to calculate the cell proliferation index (NDI) and also record the number of cells in necrosis and apoptosis as indicated in the protocol [45, 46].

4.2 Application of the cytokinesis-block micronucleus (CBMN) assay in animal species

CBMN has been used to determine genomic instability in several models. Initially developed for human lymphocytes [47], it has been tested in other animal

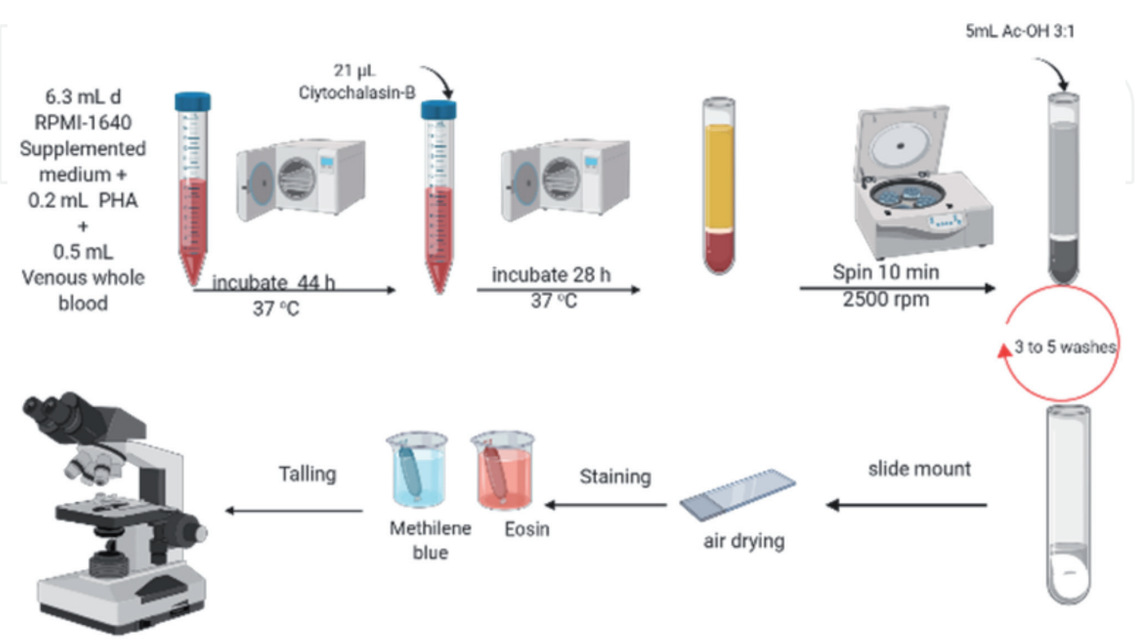

Figure 2.
The general procedure of the cytokinesis-block micronucleus assay in lymphocytes.

models of veterinary interest, such as cow [48–51], goat [52, 53], pig [54–56], rabbit [57, 58], horse [54], rodents [59], hamster cell lines [60], and rodent cell lines [61].

In most of the published articles presented in **Table 2**, only the MNi number was reported, eight included NDI, and only three took into account other nuclear abnormalities, such as NBUDs [50] and NPBs [49, 60]; no articles that considered the count of cells in necrosis and apoptosis were found.

CBMN is a valuable model for testing genomic instability effects in veterinary pharmacology experiments, such as the one performed in cows to test the mixture of an antiparasitic (cypermethrin) and a pesticide (chlorpyrifos), which reported 16.1 ± 2.3 NBUDs and found no evidence of cytogenotoxicity compared to the gamma radiation exposure [50]. The cytotoxic potential of epoxiconazole and fenpropimorph was also evaluated in bovine lymphocytes, and findings showed no genotoxic effects, however, the cell proliferation index decreased [51]. Moreover, in a trial using the antibiotic enrofloxacin [49], authors found that by increasing the dose, the number of MNi also increased. Another study with dogs analyzed the effect of oral administration of cadmium oxide (10 mg/K), where no significant differences after administration for 3 and 28 days [56] were observed. Finally, it has

Taxonomic Group Order, Family	Species, common name	n	CBPI	MNi frequency	BN cells counted	Ref
Artiodactyla Bovidae	*Bos primigenius* Taurus, cow	20	1.45	12.3 ± 4 .1	500	[48]
		3	1.57 ± 0.06	39 ± 2.5	1000	[49]
		1	1.3 ± 0.03	11.0 ± 3.2	1000	[50]
		2	1.28 ± 0.001	13.5 ± 0.71	1000	[51]
	Capra, goat	3	ND	5 ± 2	500	[53]
Artiodactyla, Suidae	*Sus scrofa*, pig	5	ND	5.8	1000	[54]
		3		8.33 ± 1.528	1000	[58]
Carnívora, Canidae	Canis canis, dog	?	ND	35 ± 4	1000	[62]
		20	1.67 ± 0.21	11.0 ± 3.29	1000	[55]
		30	ND	4.61 ± 0.88	1000	[56]
Lagomorpha, Leporidae	*Oryctolagus cuniculus*, rabbit	5	1.55 ± 0.01	6.33 ± 0.94	2000	[57]
		3	ND	5.0 ± 2.0	500	[53]
		1	ND	6.8	1000	[54]
Perissodactyla, Equidae	*Equus caballus*/ horse	N/A *	1.914 ± 0.002	16.33 ± 0.298	1000	[60]
Rodentia, Cricedae	*Cricetulus barabensis*)/ Hamster	N/A **	1.67 ± 0.016	3 ± 1	2000	[61]
Rodentia, Muridae	*Mus musculus*/ mice	6	ND	51 ± 2.16	1000	[59]
	Mus musculus/ mice L-929	N/A **	1.67 ± 0.016	3 ± 1	2000	[61]

Sample size (n), Cytokinesis proliferation block index (CBPI), Frequency of binucleated cells with micronucleus (MNi frequency), Number of binucleated cells counted (BN cells Counted).
** Chinese hamster ovary cells (CHO-K1).*
*** L-929 murine fibroblast cell line.*

Table 2.
CBMN studies in different vertebrates.

been reported that loperamide reduces cell proliferation and produces a significant increase in the number of MNi [57].

In general, it has been established that the number of spontaneous MNi in bovines is 3 times higher compared to human lymphocytes [48]. On the other hand, CBMN in goat estimates a better dosimetry fit for gamma radiation than in humans and rabbits. However, pigs and horses also show an excellent dosimetry correlation against X-rays and gamma rays [52, 54, 63].

CBMN has shown that dogs as human pets are excellent sentinels of exposure to environmental factors [55], partly because canine lymphocytes are three times more sensitive than humans to radiation [62].

5. Flow cytometry

Flow cytometry is a technique that started as an immunological technique at the beginning; however, currently, it represents a tool to perform fast and multiparametric analyses in molecular biology, microbiology, virology, toxicology, cancer biology, and infectious diseases that can affect any organism [1].

The equipment needed to perform flow cytometry is a cytometer. This is a machine capable of analyzing cells or particles mixed in a liquid solution that makes them pass one by one into tubes with a unique system of fluids. The positioning of cells in a line allows the exposure of every single cell to a laser light, which interrogates each cell individually. Then, the interpretation is performed by a computer that analyzes the light as numeric and graphical data in a standardized format (*.fcs), which can later be read and analyzed by any flow cytometry software [1, 2].

For the flow cytometry data analysis, the first step involves standardizing the studied cell population. Then, the cohort points for the negative and positive phenotypic screening molecules should be identified in the selected cell population. To reach a better identification of the phenotypic molecules, it is necessary to use a

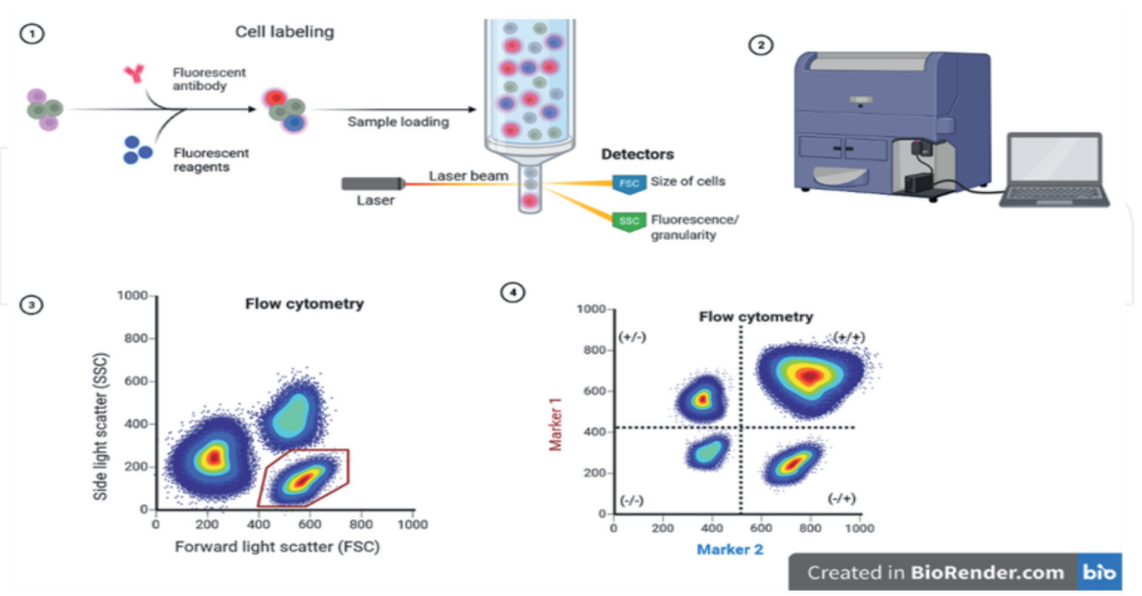

Figure 3.
Flow cytometry methodology. 1) Labelling of cells or particles with fluorescent molecules. 2) Cell mixture leaves the nozzle in droplets, laser beam strikes each cell or particle by the FSC detector, which identifies cell size, and the SCC detector, which identifies fluorescence/granularity/complexity. 3) Conversion of luminescent signals into numerical and graphical data to select the cell population according to its size and complexity. 4) Detection of fluorescent markers in cells by a pseudocolor quadrant density plot: Negative cells without fluorescence (-/-). Positive cells to fluorescent marker 1(+/-). Positive cells to fluorescent marker 2 (-/+). Positive cells to both fluorescent markers (+/+).

fluorescent positive control that can be a sample of cells from the same population with the maximal expression of the molecule; also, a negative control without a fluorescent signal should be considered (**Figure 3**) [2, 4].

To assess cell damage, the measurement of several indicators is available. In this context, cell viability is used as an indicator of cytotoxicity and involves the use of kits with contrast fluorescent colors (red and green). The viable cells will be the ones that have no damage at all, and they will be detected with a green color (495–515 nm); whereas cells with severe damage are discriminated by red brilliant (495–615 nm); the positive cells to both of the parameters, are in a process of early death, but still viable [64]. To be more specific in the cell death state, it is possible to define the apoptosis level using an annexin V/propidium iodide (PI) kit, which discriminates live cells by the absence to both fluorescent dyes; whereas the positive cells for only annexin V are in early apoptosis, while the positive cells for only PI are in necrosis; and the positive cells for both annexin V and PI dyes, are in frank or late apoptosis [65, 66].

5.1 Detection of MNi and other abnormalities by flow cytometry

Cytotoxicity and genotoxicity can be evaluated by flow cytometry. The initial approach to estimate genotoxicity by MNi detection is possible by ethidium monoazide bromide (EMA) staining to label the chromatin of necrotic and mid/late-stage apoptotic cells. In addition, stripping of cytoplasmic membranes and incubation with the pan-nucleic acid dye SYTOX Green plus RNase to provide a suspension of free nuclei also allows detection of MNi [67, 68].

Some authors have used cytometric techniques to quantify MNi in normochromatic and polychromatic erythrocytes, leading to a significant reduction of the counting time by 100 orders of magnitude and also reducing the number of experimental animals needed to perform the studies with the *in vivo* peripheral blood erythrocyte technique [67–69]. Flow cytometry is also used for counting MNi in bone marrow-derived erythrocytes and peripheral blood erythrocytes through *in vivo* experiments. Still, the most relevant advantage has been the adaptation of three approaches: flow cytometry, image recognition, and machine learning to detect both MNi and other nuclear abnormalities (NBUDs, NPBs) as well as necrotic and apoptotic cells, which opens a new perspective in the CBMN assay with lymphocytes [70–73].

6. Conclusions

The evolution of techniques that analyze genetic instability as micronuclei (MNi) and other cellular abnormalities has opened a new strategy to prevent cytogenotoxic effects on captive, farm, pet, and wild animals. On the other hand, these techniques contribute to better understand the pharmacology of drugs and the permissible environmental exposure levels to xenobiotics in laboratory studies.

BmMNt, PBMNE, and CBMN genomic instability tests have their sphere of applicability, advantages, and limitations. While BmMNt is mainly applied for *in vivo* experiments, its biomarkers are the end point, and it is not possible to follow up the effect for a long time. On the other hand, PBMNE allows daily monitoring, especially in pharmacological, toxicological, and dosimetry experiments. CBMN is one of the most comprehensive MNi-based assays. Although it can only be performed in animal models, of which, collecting at least half a milliliter of intravenous blood is possible to record six biomarkers of genomic instability: MNi, NBUDs, NPBs, NDI, and cellular death (necrosis and apoptosis).

Lastly, the flow cytometry improvements based on the synergy between flow cytometry, image recognition, and machine learning opens a new clinical scenery in micronucleus-based tests to detect genomic instability in all types of species, especially in those of veterinary interest.

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Conflict of interest

"The authors declare no conflict of interest."

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