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Review

Using the comet and micronucleus assays for genotoxicity studies: A review

Rodrigo Pinheiro Araldi [a,b,c], Thatiana Corrêa de Melo [a,d], Thais Biude Mendes [c,d], Paulo Luiz de Sá Júnior [a], Bruno Heidi Nakano Nozima [d], Eliana Tiemi Ito [c], Rodrigo Franco de Carvalho [a], Edislane Barreiros de Souza [c], Rita de Cassia Stocco [a,*]

[a] *Laboratório de Genética, Instituto Butantan, São Paulo, São Paulo, Brazil*
[b] *Programa de Pós-graduação Interunidades em Biotecnologia, Instituto de Ciências Biomédicas (ICB), Universidade de São Paulo (USP), São Paulo, São Paulo, Brazil*
[c] *Laboratório de Biologia Molecular, Genética e Mutagênese, Faculdade de Ciências e Letras de Assis, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Assis, São Paulo, Brazil*
[d] *Programa de Pós-graduação em Morfologia Funcional e Estrutural, Escola Paulista de Medicina, Universidade Federal de São Paulo (UNIFESP), São Paulo, São Paulo, Brazil*

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ABSTRACT

Physical, chemical and biological agents can act in the DNA, resulting in mutation involved in cancer. Thus, genotoxic tests are required by regulatory agencies in order to evaluate potential risk of cancer. Among these tests, the comet assay (CA) and micronucleus assay (MNA) are the most commonly used. However, there are different protocols and recommendations already published. This is the first review, after the inclusion of CA in S2R1 guidance and OECD 489, which summarizes the main technical recommendations of both CA and MNA.

1. Introduction

Even before the description of the DNA structure, it was already evident that chemical, physical and biological agents could interact with the genetic material, resulting in mutations [1–3], which are associated to genomic instability and cancer [4]. Considering this, regulatory agencies such as Food and Drug Administration (FDA), European Medicines Agency (EMA) and *Agência Nacional de Vigilância Sanitária* (ANVISA, Brazil) begun to require tests of genotoxicity as essential part of drug validation [5,6]. These tests include *in vitro* and *in vivo* assays to detect the drug potential to induce genetic mutations and/or chromosomal aberrations [7,8].

The Guideline S2 (R1) on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use is applied by FDA, EMA and ANVISA to test new drugs under development. This Guideline suggests two options of battery tests: Option 1 – test of reverse mutation in bacteria, followed of one *in vitro* cytogenetic test to evaluate chromosomal damages (chromosomal aberration or micronucleus assay) or genetic mutation test in mice lymphoma TK cell and one *in vivo* test (chromosomal aberration or micronucleus assay); Option 2 – test of reverse mutation in bacteria and *in vivo* genotoxicity evaluation in two tissues: hematopoietic (micronucleus assay) and other *in vivo* test [7–9], such as the comet assay [10]. However, the guideline also allows the use different methods, since the researcher/institution can prove the drug safety.

Among the available genotoxicity tests, comet assay (CA) and micronucleus assay (MNA) are recognized due to their robustness, sensitivity and statistical power to evaluate DNA breaks, which can be considered hallmarks of mutagenicity [11]. Furthermore, currently studies point out that the association of CA and MNA is the best battery test to evaluate the mutagenic potential, since both assays are highly sensitive, simple and allow to detect breaks at chromatic and chromosomal levels, respectively [10]. However, in function of great quantity of protocols published and the latest discovery and recommendations of both CA and MNA, it is required a review about these techniques. This review brings the latest technical considerations and possible applications for CA and MNA based on the literature and authors' expertise. Moreover, this is

* Corresponding author. Tel.: +55(11)2627 9701.
E-mail address: rita.stocco@butantan.gov.br (R. de Cassia Stocco).

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first review that brings technical aspects of these assays after the CA and MNA had been included in the S2R1 and OECD 489 guidance as battery option [12].

2. Comet assay (CA)

The CA, also known as single cell gel electrophoresis (SCGE) or microgel electrophoresis (MGE), was introduced by Östling and Johanson [13] to detect DNA damages induced by radiation. Since its development, several methodological modifications were proposed [14]. However, the alkaline method, developed by Singh et al. [15], that allows the DNA denaturation, as well as the detection of alkali-label sites, became the most used and recommended due its broad-spectrum of detection of DNA damage [14–18].

CA has been used in different studies, such as: toxicology genetics [19–21], biomonitoring [22–28], eco-genotoxicity [29–31], molecular epidemiology [32], nutrigenomics [33,34], DNA repair system studies [35–38], evaluation of nanomaterial genotoxicity [39], evaluation of DNA integrity in mesenchymal stem cell [40] and spermatozoids [41–44]. CA was also proposed to detect of bacteriophage mediated bacterial cell lysis [45] and employed in plants [46].

Since currently works point out its versatility, CA has been extensively employed in toxicological genetics studies [17,19,47–50], as it can be used as indicative of virus activity of both human papillomavirus (HPV) [51] and bovine papillomavirus (BPV) [52]. Studies involving the CA in virology have been contributed with the elucidation of viral oncogenesis mechanisms. Genotoxic action of measles virus [53] and bovine leukemia virus [54] was also reported using the assay. Thus, CA can be considered a gold standard method to study the oncogenic process associated with virus infection [55]. Due the CA versatile, the technique was currently included in the *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use* (ICH) S2R1 guidance [12].

After the standardization of the CA methodology in the *International Workshop on Genotoxicity Test Procedures* [14] and the establishment of technical recommendations on the *4th International Workshop on Genotoxicity Testing* [47], CA was adopted as part of the battery of validation tests for new drugs by pharmaceutical industries [5,56]. Thus, the *in vivo* rodent CA was validated in 2006–2012 by the Japanese Center for the Validation of Alternative Methods (JaCVAM) in conjunction with the European Center for the Validation of Alternative Methods (ECVAM), the Interagency of Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP Interagency Center of Evaluation of Alternative Toxicological Methods (NICEATM) [10].

A study with 838 drugs, analyzed by the CA, pointed out that 56.3% of them were genotoxic [56]. Other study, with 476 drugs, also analyzed by the same methodology, showed that 43.5% of them were genotoxic [6]. These data indicate the importance of the mutagenic evaluation of pharmaceutical products, as well as of new drugs candidates to enter in the market. Based on the importance of results obtained by CA to predict possible genotoxic risks, the assay was firstly proposed by the ICH and recommended by FDA and EMA in the mutagenic analysis of drugs [14,47,49]. Besides, CA is recommended a first line mutagenic test due its high sensitivity in relation to the micronucleus assay (MNA) [49].

CA can be performed with any eukaryotic cell population *in vivo*, *in vitro* or *ex vivo*, including vegetal tissue as *Allium cepa* [57]. Other advantages of the technique include: simplicity and low cost and time, since the protocol can be executed in less than 24 h [4,14,47,58–61]. The CA allows to analyze the genotoxicity in specific tissues, which are in direct contact with the tested substance or in which occur the absorption, distribution, metabolizing or excretion, allowing to detect the clastogenicity *in situ* [5,14]. The technique could be associated to fluorescent *in situ* hybridization (FISH), brings new possible of its use to analyze the DNA damage induction [62].

Due all advantages and applications of this technique, the number of publications involving the CA has grown in the last years consistently [59,63], making the comet assay a field of great interest [64,65]. PubMed registers more than 7600 citations of CA between 1990 and 2013, reinforcing the importance of this technique [65]. The database of PubMed registered 737 publications involving the CA in 2014 and 173 in 2015, since this date. In function of the greater importance of the CA, some journal dedicates special issues to the assay. The latest was published in 2015 by *Mutagenesis* [65].

CA also allows to detect breaks in DNA strands, which can be visualized by the increased migration of free DNA segments, resulting in images similar to comets, justifying the name of the assay [37,60]. There are three CA techniques available: acid, alkaline and neutral, based on the pH of the electrophoresis buffer employed. At first, it was established a paradigm that the neutral technique allows to detect double strand breaks (DSBs), whereas the alkaline technique, simple strand breaks (SSBs) [58]. However, the CA indicates both SSBs and DSBs, independently of the used technique [14,60]. These SSBs and/or DSBs are associated to chromosomal aberrations and genomic instability [66]. The genomic instability is directly associated to malignancy [67–73].

2.1. Technical principals and recommendations for the alkaline CA

The CA consists in the immobilization of a cellular suspension, homogenized with low melting point (LMPA) agarose, in pre-treated slides with normal melting point (NMPA) agarose [52,60]. The material is covered with a coverslip in order to ensure a homogeneous distribution. After the solidification, the coverslip is removed and the slides are transferred to the lysis solution [52]. This lysis solution contains cellular surfactants (Triton X-100), which remove membranes [17,60]. The slides are transferred to electrophoresis tank, being treated with a solution of sodium chloride, in a concentration greater than 2.0 M and pH >13.0 [19]. This solution promotes histone release and DNA unfolding. Under electrophoretic field, free DNA segments, product of breaks (clastogenesis), migrate in direction of the cathode, originating a comet tail [60]. After electrophoresis, the material is neutralized, fixated and stained. The slides are analyzed in fluorescent microscopy or optic microscopy, according to the employed dye [60,74]. Although different methodologies have been published, some recommendations were established to guarantee the result quality. Among these recommendations are as follow:

2.1.1. Choice of biological sample

CA can be performed in any tissue, including: whole blood [75], peripheral blood mononuclear cells (PBMCs), isolated with Ficoll-Paque or Tris–EDTA buffer (TE) [61] or culture cells [76]. However, the genotoxicity studies of chemical compounds require special attention to the age of the biological material donor. Extensive observations suggest that DNA damage accumulates with age [11,77].

2.1.2. Material conservation

Studies point out that blood conservation of 4 °C induces DNA damages, being recommended the conservation at −20 °C, −80 °C

or −196 °C [60,61,75,78,79]. Studies involving samples of fresh and cryopreserved blood confirmed that the cryopreservation does not induce DNA damage [78,79]. It is recommended that tissue samples should be preserved at −20 °C in culture medium, supplemented with 10% of fetal bovine serum and 10% of DMSO (dimethyl sulfoxide) [60].

2.1.3. Cell culture

Cell on cultivation at 37 °C promotes DNA damages [58]. So, cell destined to the CA may be incubate for at a maximum of 72 h, to avoid false-positive results [58].

2.1.4. Cellular isolation from tissue

Cellular isolation is required when sample tissues are employed. Studies show that any form of cellular dissociation, enzymatic, employing collagenase or trypsin, or mechanical is able to induce DNA damage [4,14]. Some recommendations to avoid DNA damages due the cellular dissociation are: use of trypsin at a concentration of 0.01% for cellular dissociation [58], addiction of EDTA to chelate the calcium and/or magnesium, avoiding endonuclease activation and DMSO to prevent DNA damages induced by the oxidation [14].

2.1.5. Lysis conditions

The lysis solution employed in the CA is based on Cook et al. [80], which comprises a non-ionic detergent and a high molarity of sodium chloride. The lysis removes the plasmatic membrane, cytoplasm, nucleoplasm and more than 95% of all proteins, allowing the migration of free duplex fragments [16,81]. Incubation should be performed at 4 °C to avoid possible DNA damages for a minimum of 1 h and a maximum of four weeks [60]. Addiction of DMSO in lysis solution is also required when samples of whole blood are employed to prevent DNA damages related to hemolysis [14]. After lysis, it is recommended to wash the material in distilled water to remove residues of salt and detergents that could affect electrophoresis [14]. Although the lysis time could be between 1 h to 1 mouth, it is important that each laboratory standardizes a time of lysis [82].

2.1.6. Agarose concentration

The agarose concentration is considered a critic factor for the technique [83]. It is recommended the concentration of 1.0–1.5% of NMPA agarose at 60 °C for slide pre-coverage and, 0.6–0.8% of LMPA agarose at 37 °C for homogenization of the biological material [14,60].

2.1.7. Electrophoretic conditions

It is required the incubation of the material in an alkaline electrophoresis buffer at 4 °C for 40 min to promote the release of histones [60]. Electrophoresis may be performed in following conditions: constant current of 300 mA, temperature between 4 °C and 15 °C (to avoid DNA damages), for 20 min employing 1.15 V/cm or 30 min at 0.8 V/cm [16,19,52,60]. After electrophoresis, it is recommended to wash the slides in a neutralizing buffer for 5 min, thrice [74].

2.1.8. Staining methods

Different dyes can be used in CA, including silver stain [84–86], ethidium bromide (EtBr) [19], propidium iodide (PI) [52], DAPI (4′,6-diamidino-2-phenylindole) [74,87], YOYO-1 [74] and SYBR Gold [88]. Although it is known that DAPI and YOYO-1 increase the sensitivity of DNA damage detection [74], EtBr and PI are the most commonly used dyes [17,59].

2.1.9. Material analysis

The nucleoids can be analyzed by means of automated methods or visual count and classification [37]. Automated methods are based on densitometry parameters. They identify the emitted fluorescence intensity and geometrical aspects of the nucleoids, such as tail length, head diameter and comet area [58]. The visual method consists in analyzing 100 nucleoids per slide, which are classified according to two systems: 0–2 [17,52] or 0–4 [60] (Fig. 1), where nucleoids without DNA damage are classified in 0 and, those with maximum damage in 2 or 4, depending on the system used. Based on the number of comets observed per class in a total of 100 nucleoids, the score is obtained according to the following formula: Score = $\sum i \times Ni$, where i is the DNA damage class (0–2 or 0–4) and Ni, the number of nucleoids observed per class [89,90].

The visual method is preferably eligible than the automatized method because overlapping nucleoids can be classified as a unique comet [60]. Other problem associated to the automatized systems is the recognition of hedgehog comets, cells that show elevate DNA damage, since computer programs can analyze the head as separate to the tail, classifying these comets as class zero [60]. Based on these data, the visual method is adopted preferably by most of laboratories [18,19,22,52].

2.2. Can comet assay be used to assess apoptosis?

Although the CA has been used to predict apoptosis [17,90–92], the presence of hedgehog comets, characterized by a small head and a long or absent tail, is not considered a apoptosis indicator by some authors [14,18,60].

Apoptosis is a general term commonly used to describe a no inflammatory programmed cell death in contrast with frequently non-programmed and highly inflammatory necrosis. Several features confer to apoptotic cells a peculiar signature allowing them to be discriminated from cells compromised with others

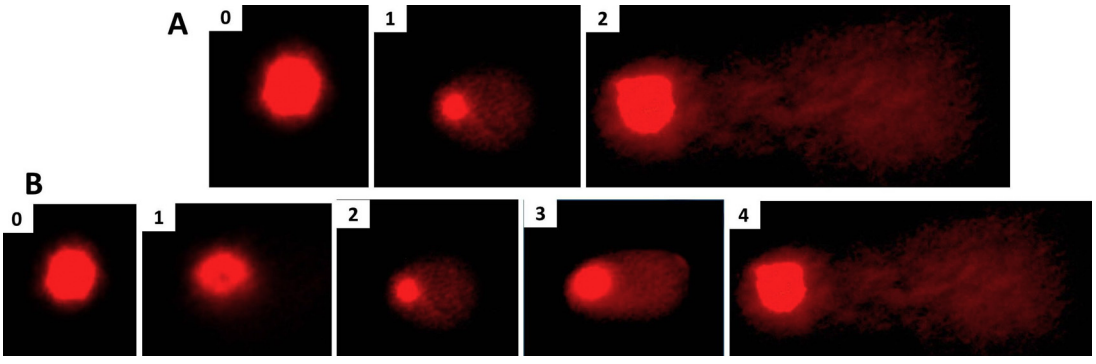

Fig. 1. Classification of nucleoids according two systems: (A) 0–2, in which 0 represent cells without DNA damage and 2, cells with maximum DNA damage and (B) 0–4, in which 0 represent cells without DNA damage and 4, cells with maximum DNA damage.

non-apoptotic mechanisms [93]. Apoptosis is characterized for a broad range of features that taken together constitute the apoptosis signature. Among all apoptotic signatures, stands out the morphological feature on which dying cells depict cytoplasm retraction, detachment from surface and pyknosis. In addition, to these morphologic rearrangements, several enzymes are concomitantly activated and act accurately in order to turn apoptosis an irreversible process. The process starts with Flippases, which are responsible for moving the phospholipid phosphatidylserine from cytosolic (inner side) to out of leaflet from cytoplasm membrane [94]. Then, the cysteine-proteases caspases family (*e.g.* CASP3, CASP6, CASP7) are responsible to deconstruct the cell framework [95]. Finally, the caspase-activated-DNAse (CAD) promotes DNA cleavage, generating fragments containing 180–200 base pair (bp) fragments. These DNA fragments are packaged into small vesicles originated from cytoplasm membrane named apoptotic bodies which, in turn, are cleaned through phagocytosis by neighboring cells, concluding the apoptotic process [96].

Cellular tests aiming to decipher the cytotoxic mechanisms by which molecules with therapeutic potential operate are widely used in drug development [93]. Thus, the choice of the techniques which must be applied will depend on the nature of the response that is sought. For instance, light morphological determinations are advantageous since is inexpensive, easy execution and rapid to perform. Disadvantages are propensity to underestimation and unsuitability for quantitative studies. Immunological methods such as cytofluorometry allows automated analyses on a per-cell basis while requires high performance of primary antibodies. Electron microscopy provides precise ultra-structural information but is expensive and time-consuming. In addition, once the methods applied to cell death characterization can be influenced by a wide range of interfering factors, results obtained by one method must be confirmed by another one [93]. Thus, currently several methods to identify DNA damages may be performed and CA stands out as a potential alternative to evaluate DNA injuries mainly due easy implementation and low costs. Many groups have been applied CA in order to evaluate DNA damages in apoptotic cells. However, albeit little is known about the mechanisms involved in DNA fragmentation in necrotic cell, it is well established that necrosis also leads to a DNA degradation [97]. In this context, the result obtained through CA *per se* does not allow characterize apoptosis since necrotic cells also presents DNA fragmentation. Therefore, we suggest that studies aiming elucidating therapeutics and cytotoxic mechanisms of chemical compounds or even radiotherapy should also include tests for determining the type of cell death (*e.g.* apoptosis, necrosis or others) as well as assays for clastogenicity in order to avoid mutual interference and misinterpretation. However, it is interesting to stress that even performing these tests for cell death and clastogenicity detection, this issue is still not totally solved since not all DNA fragments are related to cell death process described above. Dividing cells containing DNA strands breaks lacking centromeres or even entire chromosomes that do not migrate to spindle pole during cytokinesis can generate chromosomal fragments that are packaged in nuclear envelope forming structures that resembles small interphase nuclei, named micronuclei (MN) [98]. Apoptotic cells can form MN, however, the relationship between MN and apoptosis is deeply complex and new studies has to be developed in order to achieve better understanding of this imbricate issue [99].

3. Micronucleus assay (MNA)

The MNA is an important *in vivo* and *in vitro* biomarker, extensively used in the molecular epidemiology and cytogenetic damages in populations exposed to genotoxic agents [100–104]. The micronucleus (MN) term, also known as Howell–Jolly bodies [105], was introduced in 1951 related to acentric fragments expelled from the main nucleus at late stages of anaphase [106]. MNs can be formed through two mechanisms: chromosomal breaks (clastogenesis) or disruption of the mitotic apparatus (aneugenesis) [101,103]. Thus, using anti-kinetochore antibody it is possible to verify if a particular drug induces MN formation *via* clastogenesis or aneugenesis [105]. Absence of kinetochore in the MNs indicates clastogenic action, whereas its presence, aneugenic action [105].

MNs are formed along the erythropoiesis, which occurs in the bone marrow or spleen of adult rodents [105]. Erythroblasts excluded the nucleus after 6 h of the final mitosis [107], originating polychromatic erythrocytes (PCE), basophilic cells that contain RNA in the cytoplasm detectable by Giemsa stain [105]. The PCEs suffer a maturation process, originating normochromic erythrocytes (NCEs), acidophilic cells that stain orange or orange-pink with Giemsa [105]. Thus, clastogenic and/or aneugenic agents are able to originate chromosomal fragmentation or chromosomal losses during the cellular division that are not integrated in the nucleus of daughter cell, resulting in MNs [98,105]. These MNs are enveloped by nuclear membrane during the telophase [98] and can be visualized in the cytoplasm [105]. An elevate frequency of micronucleated PCEs (MNPCEs) indicates chromosomal damage [98,105,108].

However, the MNs not only represent chromosomal losses, but also the result of DNA amplification [103]. DNA amplification is commonly observed in oncogenic process, resulting in double minute chromosomes (DM), which are removed from the main cell nucleus, originating MNs [103,109]. The MNs expelling is associated with the loss of allele dose, contributing to carcinogenesis [101,102]. A scheme of the micronucleus formation is shown in Fig. 2.

Since 1959, the MNs have been proposed as marker of cytogenetics damages [106]. However, the frequency analysis of MNs as a cytogenetics test was only proposed in 1970 by Boller and Schimid [110] and latter employed in polychromatic erythrocytes of bone marrow [111] and lymphocytes [112]. The MNA has been widely used in studies of genotoxicity [113–115]. MNA can be applied to any eukaryotic cell, being preferable used in substitution of the chromosomal aberration test, because it does not require karyotype analysis [108,114,116,117].

MNA, as well as the comet assay, has been used in virology field. Studies point out that the Tax protein of Human T-Leukemia Virus type I (HTLV-I) and II (HTLV-II) induces the increase of MN frequency [118,119]. Similar results were also observed with the Epstein-Barr Virus (EBV) [120]. Currently studies have been demonstrated that HPV is related with the increase frequency of MN in both cytology and peripheral blood of women infected by the virus [121,122]. Duensing and Münger [123,124] demonstrated that the E6 and E7 oncoproteins of HPV are directly associated with the MNs induction. Other study developed for our group pointed out that PBMCs and epithelial cells of bovine kidney (CRIB) treated with 1 μg/ml of recombinant E6 oncoprotein of BPV-1 show elevated frequency of MN in relation to not treated cells (data not published).

Some advantages of MNA over CA are: it only considers genetic damages in mitotic cells, whereas CA detects DNA damages in both interphase and mitotic cells [125], the MNA has a greater statistical power, since it analyze over 1000 cells, whereas the CA analyze 100 cells [125]. Although the MNA has advantages in relation to the chromosomal aberration test and CA, studies in toxicology genetic reinforces the necessity of more than one mutagenic test [19,89,126]. Thus, the association of MNA and CA can be considered as a gold standard among mutagenic tests, because

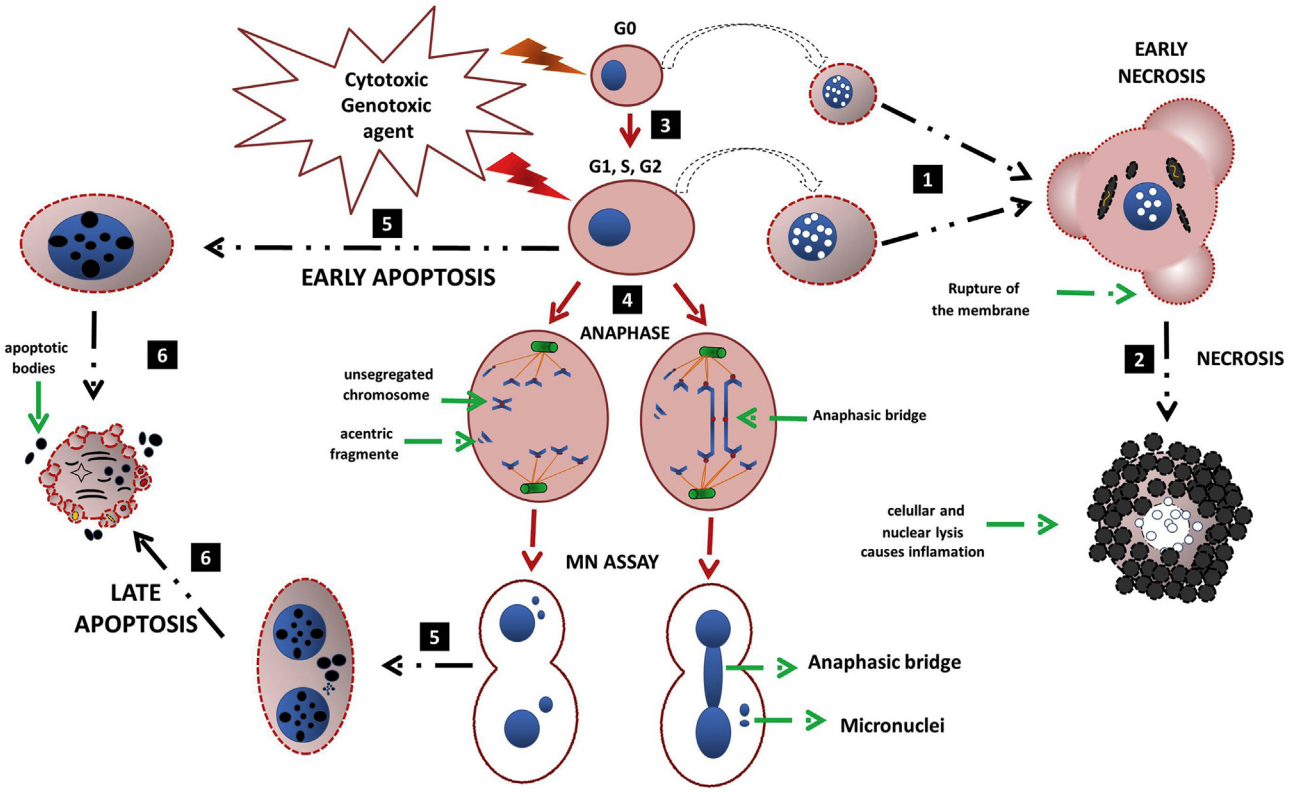

Fig. 2. Different pathway of mutagenic drugs. Cytotoxic agents induce an early necrosis phase (1), which evolves to necrosis (2). Genotoxic agents induce DNA damages during the G_0 to S phase (3), which can induce apoptosis (5), resulting in apoptotic bodies formation, during the late apoptosis (6) or, leave to chromosomal damages, resulting in micronucleus formation through the chromosome breaks and/or disruption of mitotic apparatus (4). Micronucleated cells can be destined to apoptosis (5 and 6).

they have high sensitivity, statistical power, being simple, versatile, and demands low cost of time and investment. MNA can be performed in polychromatic erythrocytes (*in vivo*) [19,104] and cells culture of both lymphocyte [103] or cells lineages (*in vitro*) [127]. Images of cells micronucleated obtained with both *in vivo* and *in vitro* MNA are shown in Fig. 3. The *in vitro* technique requires the cytokinesis blocking, being known as cytokinesis-block micronucleus assay (CBMNA) [108].

The MNA's protocol was standardized by means of an international consortium between pharmaceutical companies F. Hoffmann-La Roche, Novartis, Rhône-Poulenc Rorer and Biologie Servier [117]. This consortium pointed the MNA as indicated to the genotoxicity evaluation of drugs [117]. Furthermore, the MNA was elect by the *International Workshop on Genotoxicity Test Procedures* as gold standard test in mutagenesis [106]. The main recommendations for both *in vivo* and *in vitro* MNA are discussed in this review.

3.1. Technical recommendations for the in vivo micronucleus assay (MNA)

The *in vivo* MNA is preferably recommended in relation to the *in vitro* MNA because it allows to evaluate the drug metabolizing, pharmacokinetics and DNA repair [105,128]. The *in vivo* MNA is the first assay in the battery of genotoxicity tests accepted by agencies such as FDA and EMA [105,129].

3.1.1. Species selection

Although any rodent can be used for the *in vivo* MNA, without preference for strain, rats and mice are the most commonly species employed [19,104,130]. However, studies pointed out that the spleen of rats and humans are able to remove the MNPCEs of the blood circulation [131,132]. For this reason, the MNPCEs frequency analysis in peripheral blood is only accepted for mice [128].

It is recommended the use of animal aged to 6–8 weeks [19,129], of both sexes, once different responses of metabolizing, toxicity or pharmacokinetics can be observed according to the sex [19,107,130]. The animal may be acclimated at a minimum period of five days in following conditions: food and water *ad libitum*, light–dark cycle of 12:12 h and a temperature of $22 \pm 2\,^{\circ}C$ [19,105,133].

3.1.2. Dose level and administration pathway

This is recommended a limit dose of 2 g/kg/day for treatments of 14 days or less or, 1 g/kg/day for treatments period greater than 14 days [105,130]. The tested drug(s) may be dissolved in water or isotonic saline, for hydrophilic compounds, or vegetable oil for hydrophobic substances [130]. The drugs can be administrated by oral gavage, subcutaneously or intraperitoneally [130,133].

3.1.3. Controls

The *in vivo* MNA requires a negative, positive and experimental groups, each one with a minimum of five animals [130]. As negative control, the drug solvent may be used [19,130]. Positive control is required in all assays of toxicology genetics [129]. Different drugs have been used as positive control. Some examples of drugs and their respective concentrations used as positive control for mice are: 200 mg/kg ethylmethanesulfonate (CAS 62-50-0), 50 mg/kg N-ethyl-N-nitrosourea (CAS 759-73-9), 50 mg/kg mytomicin C (CAS 50-07-7) and 40–50 mg/kg cyclophosphamide (CAS 50-18-0) [19,105,133]. Positive control may administrated by a different pathway of the tested drug, if possible [105].

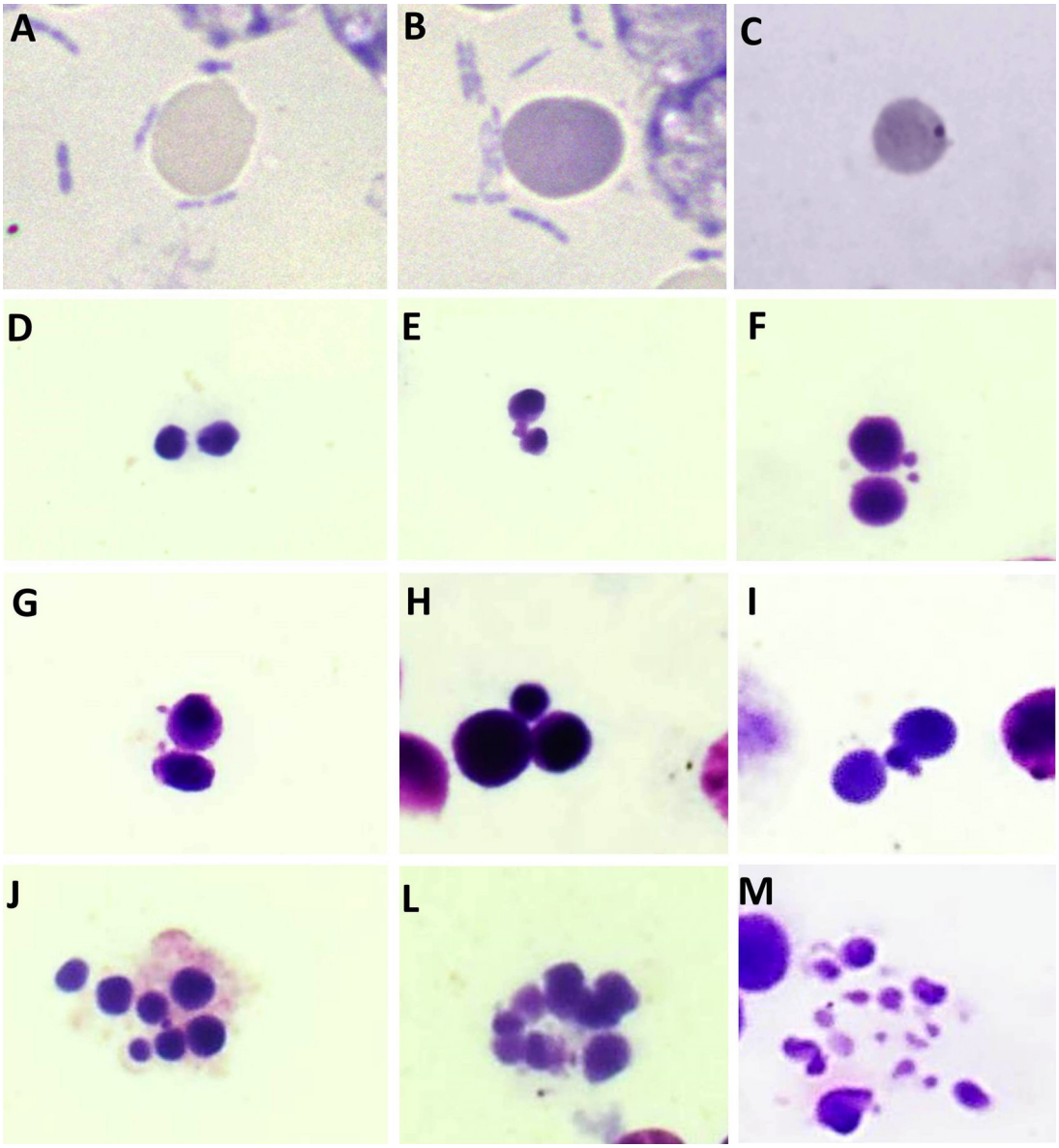

Fig. 3. Possible results observed with both *in vivo* (A–C) and *in vitro* (D–M) micronucleus assay. Images of erythrocytes, obtained with total magnification of 1000×: normochromatic (A), polychromatic (B) and polychromatic with micronucleus (C). Images binucleated lymphocyte, obtained using Cyt-B in a total magnification of 1000×: normal, without chromosomal damage (D), with nucleoplasmatic bridge (E), showing two micronucleus (F–I). Images of apoptotic bodies induce (J–M).

3.1.4. Bone marrow obtainance

The bone marrow may be collected 24–48 h after the single dose administration or 18–24 h after the last application of the tested drug, for substances administrated more than once a day [105]. A total of 2000 PCEs may be analyzed, being observed the frequency of MNPCEs [130]. MNs can be stained with acridine orange [134,135], Giemsa [136], Hoechst 33258 and pyronin-Y [130].

3.1.5. Micronucleus analysis by flow cytometry

The use of flow cytometry was standardized in the 4th International Workshop on Genotoxicity Test Procedures, representing an advance in the MNA [128]. The flow cytometry allows to analyze a greater number of cells, which increase the statistical power of the technique, as well as its sensitivity [128]. This technique requires the use of anti-CD71 antibody and propidium iodide for MNs staining [131,132]. The CD71 is a marker of erythrocyte maturation, which is present is PCEs [131].

3.2. Technical principals and recommendations for the in vitro *micronucleus assay (CBMNA)*

The CBMNA is an *in vitro* technique used to evaluate the genotoxicity potential in lymphocytes or cell culture. The technique requires the use of cytochalasin B (Cyt-B). The Cyt-B is a metabolite, obtained from the fungal *Drechslera dematioidea*, that inhibits both the rate of actin polymerization and the interaction of actin [137], blocking the cytokinesis without causing DNA damages [100,108]. The Cyt-B may be added after 24 h of lymphocyte culture start or 1 h after the cells synchronization at a maximum concentration of 0.01 M [138]. The cell synchronization can be performed through the cell culture without fetal bovine serum [139,140]. The use of Cyt-B in lineage cells is optional, once genotoxic agents induce the formation of MNs *per se* [106]. Despite considered optional, its use allows to evaluate the presence of nucleoplasmatic bridges [106]. These bridges result from dicentric chromosomes in which the two centromeres are pulled to opposite

poles of the cell, indicating chromosomal rearrangements [98]. However, the Cyt-B use is obligatorily required in lymphocyte culture, being its use recommended in the following concentrations: 3 μg/ml for whole blood and 6 μg/ml for culture of lymphocyte isolated with Ficoll-Paque or TE [106]. Cyt-B may be dissolved in DMSO [98,141] and, applied after 44 h to lymphocytes culture [108] or 1 h after the cell synchronization of lineage cells [100,127,142].

For the CBMNA, the time of cell culture cannot exceed 72 h [114] and the dose of tested drug cannot exceed 10 mM [106]. A total of 1000 cells may be analyzed per slide [106]. Based on the ratio between the micronucleated cells (A) observed and the total of binucleated cells (B), it is possible to calculate the frequency of MNs formation (MN_{r0}) by the formula: $MN_{r0} = A/B$ [127]. It is also possible to calculate the index of proliferation with blocking cytokinesis (IPBC). The IPBC can be obtained by the formula: $IPBC = [(1 \times N_1) \times (2 \times N_2) \times (3 \times N_3)]/1000$, where N_1 is the number of mononuclear cells, N_2, number of binuclear cells and N_3, number of trinuclear cells.

4. Conclusion

In summary, the CA and MNA together allow to detect aneugenic and clastogenic substances with high sensitivity and statistical power. The use of both tests allows also evaluating the systemic or *in situ* genotoxicity, once both assays can be employed in specific tissues or cells. Thus, the introduction of the recommendations summarized in this review, based on others studies and reviews, by regulatory agencies, such as ANVISA, should be considered. These recommendations are important to guarantee the reliability of results.

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