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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