The Comet assay and the troubles with its application in the green alga *Chlamydomonas reinhardtii*.

Kométový test a problémy s jeho aplikáciou u zelenej riasy *Chlamydomonas reinhardtii*.

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Abstract

The single cell gel electrophoresis or Comet assay is a sensitive, reliable, rapid and economic method for DNA double- and single-strand breaks, alkali-labile sites and delayed repair site detection, in eukaryotic individual cells. Given its overall characteristics, this method has been widely used over the past few years in several different areas. In this paper we review briefly the basic data about the principles, methodology and applications of this assay and we discuss also the possible explanation for not achieving comets to date in single-cell green alga *Chlamydomonas reinhardtii*.

Introduction

Genomic integrity is under constant threat in all species. These threats come in many forms (e.g., agents that damage DNA, spontaneous chemical changes, and errors in DNA metabolism), lead to a variety of alterations in the normal DNA structure (e.g., single- and double strand breaks, chemically modified bases, abasic sites, bulky adducts, inter- and intra-strand cross-links, and base-pairing mismatches) and have many direct and indirect effects on cells and organisms (mutations, genetic recombination, the inhibition or alteration of cellular processes, chromosomal aberration, tumorigenesis, and cell death).

There are many methods available for the scoring of DNA damage and repair. Up to now the most commonly used are the bacterial Ames test, the scoring of chromosome aberrations, micronuclei and sister chromatid exchanges (SCE) in proliferating cell populations and, for DNA repair studies, the

detection of DNA repair synthesis with the unscheduled DNA synthesis assay. Many high-throughput screening tests such as the 'SOS' chromotest measure cellular DNA damage response more directly. Sophisticated systems in genetically tractable organisms like *S. cerevisiae* and *Drosophila* were also developed that could rate multiple endpoints in the same strain, including mitotic crossing-over and gene conversion as well as chemically induced mutagenesis. Recently a battery of standardized genotoxicity tests was harmonized for use in most jurisdictions (BARCLAY 2002).

These methods are used for laboratory investigations as well as for human biomonitoring and for investigations of environmental pollution (genotoxicology testing of environmental samples and studies in different species living in the particular environments). Furthermore above-mentioned techniques were also used to investigate the anti-carcinogenic/anti-mutagenic properties of natural products (GIRI et al. 1999, IKKEN et al. 1999, UMBUZEIRO-VALENT et al. 1999). These methods have been and remain very useful but nevertheless they have a number of important shortcomings (TICE 1995). Because of the need of more rapid short term screening tests, further tests were developed in recent years and others are still in development.

The Comet assay

"Comet Assay" or "Single Cell Gel Electrophoresis (SCGE) assay" is now considered a very important alternative for the cytogenetic tests; it is much less labour intensive, more rapid and less expensive. The use of the Comet Assay in eco- (geno-) toxicological studies becomes more common as scientists begin to realize the importance of the genetic damage caused by pollutants.

Development of the Comet assay

RYDBERG & JOHANSON (1978) were the first to directly quantitate DNA damage in individual cells after gamma-irradiation by lysing and embedding them in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. The cells were stained with acridine orange and the extent of DNA damage was measured by the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence. To improve the sensitivity for detecting DNA damage in isolated cells, ÖSTLING & JOHANSON (1984) proposed that strand breaks would enable DNA loops to stretch out upon electrophoresis, so the microgel electrophoresis technique was developed. Electrophoresis acted to pull negatively charged damaged DNA away from the nucleoid towards the anode and resulted in characteristic images that looked like a comet with head and tail. This technique permitted the detection of double-

stranded DNA breaks only. The microgel method was progressively improved for sensitivity and reproducibility for detecting single-strand breaks. More complete protein lysis was accomplished, and alkaline treatment step was included before or during electrophoresis (OLIVE et al. 1990a, SINGH et al. 1988). Alkali caused denaturation of the duplex DNA, and allowed the individual strands to separate and migrate independently. The name "Comet assay" was introduced (OLIVE 1989) and the application of the first image analysis program was described in 1990 (OLIVE et al. 1990b). Image analysis has become essential for objective measurement of low-dose effects, or for distinguishing small differences among sub-populations of cells. Microscopic examination (COLLINS et al. 1997) remains useful for observing larger differences (e.g. screening drugs or measuring the percentage of apoptic cells).

Detection of DNA damage

While the general steps for single-cell gel electrophoresis are fairly well defined, in the past years, the Comet assay has had several modifications but the underlying principles are based on the neutral (Olive's) and alkaline (Singh's) version. In neutral pH, only the detection of double-strand breaks is possible, since at this pH, DNA base pairing is not disrupted and thus the discontinuities in single-strand breaks cannot be detected (MCKELVEY-MARTIN et al. 1993). Alkaline conditions (pH>13) enables detection of not only frank strand breaks but also alkali-labile sites, DNA crosslinking, and transient DNA strand breaks arising due to DNA repair processes (SINGH et al. 1988). More recently, the assay was modified further to enable the detection of specific kinds of DNA damage by combining the assay with the use of a purified DNA repair enzymes, which recognize the lesions along the DNA and convert them into the DNA single-strand breaks expressed as an increase in comet DNA migration (COLLINS et al. 1993, 1996, 1997). For this purpose various DNA repair enzymes are used, especially Fapy-DNA glycosylase (FPG) and endonuclease III (Endo III) recognizing oxidized bases (COLLINS et al. 1993), T4 endonuclease for determining the relative amounts of pyrimidine dimmers (GEDIK et al. 1992), UV-DNA damage endonuclease (UVDE) or uracil glycosylase (UDG).

The Comet assay has technical variables affecting its sensitivity, the main ones are: the composition and pH of the lysing solution; the composition and pH of the electrophoretic buffer; and the electrophoretic conditions basically voltage, amperage and unwinding length and running time (for review see FAIRBAIRN et al. 1995, MCKELVEY-MARTIN 1993, ROJAS et al. 1999, ROSS et al. 1995, TICE 1995).

For example, the modification of the Comet assay described by ANGELIS et al. (1999) on plant systems employs various combinations of neutral and alkali pH solutions immediately prior or during electrophoresis. Exposure of DNA to high alkali prior to electrophoresis under neutral conditions (so called A/N protocol) allows for the preferential detection of DNA SSBs. The majority of

alkali labile sites become detectable when electrophoresis is performed in alkaline solution (A/A protocol). DSBs cause comet formation even under completely neutral conditions (N/N protocol).

Why to use the Comet assay

Generally, the Comet assay is an economic, fast, sensitive, reliable, and rapid method. Advantages of the Comet assay for assessing DNA damage includes: (1) damage to the DNA in individual cells is measured; (2) only small number of cells are needed to carry out the assay (< 10, 000); (3) it is as sensitive method for detecting DNA damage than conventional cytogenetic tests in detecting low levels of exposure (COLLINS et al. 1996, LEROY et al. 1996, LEE & STEINERT 2003); (4) data can be generated very quickly by visual scoring (COLLINS et al. 1993) and (5) the assay can be performed on virtually any eukaryotic cell type. This technique can be applied to proliferating and non-proliferating cells and the cells of those tissues, which are the first sites of contact with mutagenic/carcinogenic substances. What makes this assay even more valuable is the specificity for detecting genotoxicity. Like in other tests, DNA effects induced due to cytotoxicity is a big issue. Data show that cytotoxic effects can be detected (dead cells show specific kinds of comets called "clouds") and distinguished from genotoxic effects, therefore, should have no confounding effects on results (HARTMANN & SPEIT 1995, HENDERSON et al. 1998, SPEIT et al. 1998). Given its overall characteristics, this method has been widely used in several different areas. On the other hand, the Comet assay is not without shortcomings: (1) the majority of the DNA lesions detected by Comet assay can be repaired by cell before being fixed as mutations; (2) it's rate limiting and also a sample bias due to the small cell sample; (3) there is no single appropriate comet parameter capable of adequately describing the observed damage, so the interpretation of results might be difficult (HARTMANN 1999); (4) there are also wide variations in the methodologies followed during alkali treatment and electrophoresis (KASSIE et al. 2000).

Applications of the Comet assay

The major applications of the Comet assay are in the following areas: (1) genotoxicology – to evaluate *in vitro/in vivo* genotoxicity of several chemicals; (2) clinical area – to investigate the consequences of certain pathological conditions or therapeutical exposure to chemicals at the cellular level; (3) DNA repair investigations – to reflect the types of DNA lesions and the DNA repair that is taking place in the damaged cells; (4) environmental biomonitoring (aquatic, terrestrial) and (5) human biomonitoring (aging, nutrition, malnourishment, exercise) (for review see: ANDERSON et al. 1998, ROJAS et al. 1999, TICE et al. 1995). The Comet assay was widely and successfully used in vertebrates, especially in mammalian cells and cells from invertebrates (SALAGOVIC et al. 1996). Plant genetic assay systems are also excellent *in situ*

environmental monitors and some studies have been published also on the use of the Comet assay in *Vicia faba* (KOPPEN & VERSCHAEVE 1996), onion (NAVARRETE et al. 1997), tobacco (GICHNER & PLEWA 1998), barley (JOVTCHEV et al. 2001) or *Arabidopsis* (MENKE et al. 2001). MILOSHEV et al. (2002) detected DNA damage in the yeast *Saccharomyces cerevisiae* and IWAHORI et al. (1999) applied this assay to *Euglena gracilis*.

Why to use *Chlamydomonas*

In addition to bacterial, higher plant and animal biomarkers, there are some fundamental purposes to use the unicellular green algae to monitor for environmental mutagens: (1) unicellular green alga *Chlamydomonas reinhardtii* is an excellent biomarker for a detection of environmental pollutants, mainly in aquatic environments; (2) algae can be used as an auxiliary indicator of damaged plant ecological systems; (3) the collection of repair-deficient strains of alga *Chlamydomonas reinhardtii* is available (COX & SMALL 1985, DAVIES 1967, MIADOKOVÁ et al. 1994, PODSTAVKOVÁ et al. 1991, 1992, ROSEN et al. 1980, SMALL 1987, VLČEK et al. 1987, 1991, 1995) and some repair-deficient strains can be used for genotoxicity assessment of environmental chemicals by the procedure analogous to "repair-test" in bacteria (MIADOKOVÁ et al. 1995); (4) algae may store and metabolize non-mutagenic aquatic pollutants to mutagenic products which may be introduced into human food chain (VLČEK et al. 1997). These algal metabolites may differ from products formed by the metabolic processes in other targets, e.g. animal and higher plant cells (MIADOKOVÁ et al. 1998).

Troubleshoots in *Chlamydomonas* Comet assay

ERBES et al. (1997) applied the microgel electrophoresis technique first time to a unicellular green alga *Chlamydomonas reinhardtii* to detect DNA damage caused by genotoxins. For this, the test protocol described by SINGH et al. (1988) was modified. Major modifications were the use of alkaline lysis buffer with ionic detergents and the reduction of preincubation in alkali and electrophoresis times. Unfortunately, this protocol had not been reproducible (PANÁKOVÁ 2001) and no other papers have been reported yet. We suppose that the technical difficulties in *Chlamydomonas* assay could be caused by three main problems: (1) the cell wall and the lysis of the cell; (2) generating comet (DNA migration) and (3) visualization/quantification of DNA damage.

The cell wall and the lysis

The cell wall of *Chlamydomonas reinhardtii* is very rigid, tough and highly insoluble, consisting of seven layers and containing mainly the hydroxy-proline rich glycoproteins. Especially the inner layers of the cell wall are highly resistant (ADAIR & APT 1990, HARRIS 1989, IMAM & SNELL 1988, VOIGHT 1988), so that the lysis solutions used in experiments with other organisms are

not sufficient in the case of *Chlamydomonas*. Alkaline lysis, which is the most frequently cited in the literature, consists of immersing the cells in a high salt solution with non-ionic detergent at pH of 10 to >12 for at least 1 hour. Similar lysis results are obtained using N-laurylsarcosine in detergent mix or PK to remove any residual protein. The troubles with cell lysis were raised also in the higher plants, but these difficulties have been overcome by using isolated nuclei instead of whole cells in the Comet assay (ANGELIS et al. 1999). Nuclei were liberated mechanically by gentle slicing of any plant tissue (roof, leaves, meristems) using fresh razor blade and dipping the tissue repeatedly in cold Sörensen buffer on ice so that the isolated nuclei could be collected in the buffer and then microfiltrated (STAVREVA et al. 1998, GICHNER et al. 1999, GICHNER et al. 2000). POLI et al. (1999) have been reported another modification resulting in an increased yield of plant nuclei and a more uniform distribution of nuclei in the agarose layer.

In *C. reinhardtii* it seemed to be necessary to combine the cell lysis and removal of the cell wall with autolysine (PANÁKOVÁ 2001). Autolysine is gametic metaloprotease released by cells of *Chlamydomonas* themselves during agglutination to degrade the cell wall of the opposite mating type gamete and this enzyme is frequently used in molecular and biochemical methods (BUCHANAN et al. 1989, JAENICKE et al. 1987). On the other hand, immersing the cells fixed in the agar on the slide to the autolysine lead to the heterogeneous results and so it appears to be less effective than applying this enzyme to the cell suspension directly prior their immobilization (*unpublished results*). This mode (employing autolysine) takes much time and reduces the sensitivity of the assay, because the transient repair sites induced by tested genotoxic agent and detected by the Comet assay could be repaired to some extent during autolysine treatment, which require at least 1-2 hours. To obviate this problem and step aside the autolysine, PANÁKOVÁ and coworkers used stronger alkali lysis solution (pH ~ 13-14) according to VALLE et al. (1981), containing ionic detergent N-laurylsarcosine and by this way the complete cell lysis was achieved subsequently.

Generating comet /DNA migration

Possible explanation for not achieving the real comets in *Chlamydomonas* to date is that physical or biological barriers exist in nucleus. Little is known about the composition and interaction of nuclear matrix, protein scaffold and DNA. The fact, that the comet tail formation is so rare in the case of *C. reinhardtii*, can be explained by such interactions. Most investigation of structural components of the nucleus has focused on proteins in vertebrates and *Drosophila* (MEIER et al. 1996). Significantly less information is available for other eukaryotes and for algae in particular. The association of some plant nuclear protein with isolated nuclei and the nuclear matrix has suggested that they are attached to additional proteins that form a rigid structure lining the

nuclear envelope. Upon nuclear matrix preparation, this structure collapses from the nuclear periphery toward the nuclear interior but stays connected with the material representing the insoluble nuclear matrix. This indicates the presence of tightly linked nuclear suprastructure that include nuclear pores, the lamina, and the filaments of internal nuclear matrix in higher plants (MEIER et al. 1996, GINDULLIS et al. 1999). Recent characterization of the nuclear architecture of the *C. reinhardtii* (COLÓN-RAMOS et al. 2003) revealed a polarized nucleus, with nuclear pore complexes preferentially concentrated at the posterior side of the nucleus while heterochromatin polarized to the anterior side. This phenomenon has significant effects to cytoplasmic processes (COLÓN-RAMOS et al. 2003) and may also interfere with the comet tail formation in this alga.

Also the stage of *Chlamydomonas* cell cycle can affect the success of the Comet assay. Some papers reported that in S-phase animal/mammalian cells, the replication structures could inhibit DNA migration during gel electrophoresis in neutral conditions (OLIVE et al. 1991, OLIVE et al. 1993). From this aspect of view, only the synchronous cultures of *Chlamydomonas* cells should be used in the test. Also the interconnection between the cell cycle and the topology of cellular organelles especially the cup-shaped chloroplast, which occupies the space between the nucleus and inner membrane of the cell and surrounds the nuclear envelope, could play an important role. This cell material in relation to shortened interval of treatment in modified protocols may prevent a rapid diffusion of some chemicals using in the assay to the nuclei of embedded cells and by this a virtual trouble could raise. For example, to visualize DNA by fluorescent dye acridine orange, RNA has to be removed from lysed cells by enzyme RNase although the slides are immersed to the strong alkali solution prior their staining. This is evidence, that the treatment with alkali solution is not sufficient to degrade RNA because of short duration of lysis step (just 5 min). Unfortunately, little progress has been made to shed light on these associations.

Visualization/quantification of DNA damage

Although we are able to isolate the nucleoids of *Chlamydomonas*, the true comets have not been presented still. To overcome this problem and visualize the DNA damage, the acridine orange could be applied at the present time instead of commonly used fluorescent dyes. Acridine orange fluoresces red with ss nucleic acid and yellow/green with ds nucleic acid. The special evaluating of DNA damage have been proposed by categorizing "comets" on the basis of the ratio of yellow/green and red fluorescence. However this system of evaluation has reduced analytical sensitivity and we plan to improve described method and introduce another modifications of particular steps for comet tail formation.

Setting up the Comet assay in our laboratory could allow us to engage in three areas: (1) DNA repair mechanisms, (2) genotoxicity and (3) nuclear processes of alga *Chlamydomonas*. The main application is in studying DNA

repair, the ability some cells have to repair their own damaged DNA. The Comet assay, because it quantifies DNA damage, is the first widely available test that can monitor DNA repair. In our laboratory, the green alga *C. reinhardtii* is utilized as the model organism for the study of repair systems. In comparison with other lower eukaryotes there has been much less progress in understanding the repair processes made in algae. Optimalization of the Comet assay protocol in our conditions could serve as an effective means to investigate the repair capacity of previously isolated repair-deficient strains of *Chlamydomonas* in relation to various mutagenic agents. Another application of the novel method is in the area of genetic toxicology and in evaluating *in vitro*/*in vivo* genotoxicity of several chemicals or radiation. We suggest that the Comet assay could be beneficially used at many sites to determine if there are linkage between DNA damage and effects at the population and community levels. The alkaline single-cell gel electrophoresis assay can be combined with fluorescence *in situ* hybridization (FISH) methodology to investigate the localization of specific gene domains within an individual cell. The position of the fluorescent hybridization spots in the comet head or tail indicates whether the sequence of interest lies within or in the vicinity of a damaged region of DNA. Either whole gene or chromosome can be used to assess the gene or chromosome specific processes. Undoubtedly, there is much to be discovered in this field.

In this overview we have outlined some long-standing questions in the *Chlamydomonas* Comet assay methodology. We hope, that the proposed discoveries in our work could shed light on this area in the near future and we will able to put adequate answers.

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