Mutation Research 770 (2014) 16–22

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

# Some causes of inter-laboratory variation in the results of comet assay

Nikolai P. Sirota[a,*], Aliy K. Zhanataev[b], Elena A. Kuznetsova[a], Eugenii P. Khizhnyak[a], Elena A. Anisina[b], Andrei D. Durnev[b]

[a] Institute of Theoretical and Experimental Biophysics of Russian Academy of Sciences, Pushchino, Russia
[b] Zakusov Research Institute of Pharmacology of RAMS, Moscow, Russia

ARTICLE INFO

*Article history:*
Received 13 March 2013
Received in revised form 16 May 2014
Accepted 18 May 2014
Available online 27 May 2014

*Keywords:*
Comet assay
X-ray
DNA damage
MMS
Bone marrow cells
Alkaline electrophoresis

ABSTRACT

We performed an inter-laboratory study to determine the variation of comet assay results and to identify its possible reasons. An exchange of slides between Labs in different stages of the comet assay protocol was performed. Because identical slides, durations of alkali treatment and electrophoresis, and similar electric field strengths (2.0 V/cm and 2.14 V/cm) were used, we concluded that the observed inter-laboratory difference in the results is directly associated with the electrophoresis step. In Lab 1, mouse bone marrow cells were exposed to methyl methanesulfonate at concentrations of 10, 25 and 50 μM for 3 h at 37 °C. In Lab 2, cells the same as in Lab 1 were immobilized in LMA on slides and exposed to X-rays at doses of 3–8 Gy. We found that the transportation of slides after lysis or electrophoresis step, as well as different dyes used for scoring did not produce any significant effect on the results. No substantial difference in the data was also revealed when various software packages were used for image analysis. The temperature of the alkaline solution was shown to increase during electrophoresis and, besides, the temperature heterogeneity of the solution took place in the area of the platform, with a maximum in the middle of the chamber. The temperature heterogeneity could affect the rate of conversion of alkali labile sites into single stranded breaks. Thus, it was clearly indicated that real temperature variations during the alkali treatment and electrophoresis were an essential factor in the variability of the results between our Labs.


## 1. Introduction

Comet assay is a rapid, simple and sensitive technique for assessing and measuring DNA damage, with wide application under *in vitro* and *in vivo* conditions in studies on a variety of organisms [1,2]. Comet assay finds use as a test for evaluating genotoxicity; some regulatory authorities have recommended the assay as an *in vivo* alternative to the conventional unscheduled DNA synthesis test [3]. The International Working Group on Genetic Toxicology Testing concluded that the combination of micronucleus test and comet assay and their integration into acute and repeated dosing toxicity studies are both feasible and scientifically justified [4]. The comet assay could be suitable for use in clinical practice, in key areas of human nutrition and biomonitoring [5,6].

The validation of the comet assay posed the problem of standardizing protocols and data analysis. The results of a recent inter-laboratory validation study of ECVAG have demonstrated that there is still a need of further trials to clarify the reasons of the inter-laboratory variation [7]. It is generally accepted that the inter-laboratory variation arises from nonidentical experimental conditions and systems of analysis [8]. It has been shown that different parameters of comet assay protocols, such as low-melting point agarose (LMA) concentration, alkali treatment and electrophoresis conditions, comet staining and scoring, cause variances [9,10].

We performed an inter-laboratory study to determine the influence of the alkaline electrophoresis step on the variation of comet assay results and to identify its possible reasons. Using the same untreated and treated cells and exchanging slides between labs after lysis, electrophoresis and neutralization steps, and recording temperature changes during unwinding and electrophoresis in the laboratories, the results clearly indicated that there are real temperature variations during alkali treatment and electrophoresis which are an essential factor in the discrepancy between the results of our Labs.

## 2. Materials and methods

The studies were carried out in the Laboratory of pharmacology and mutagenesis of V.V. Zakusov's Research Institute of Pharmacology of RAMS (Lab 1) and in the Laboratory of Radiation Molecular Biology of ITEB RAS (Lab 2). The general scheme of the study is given in Fig. 1. According to the design, 12 slides were prepared in

* Corresponding author at: Institute of Theoretical and Experimental Biophysics of Russian Academy of Sciences Pushchino, Moscow Region 142290, Russia. Tel.: +7 4967 739234; fax: +7 4967 330553.
*E-mail address:* sirota@iteb.ru (N.P. Sirota).

http://dx.doi.org/10.1016/j.mrgentox.2014.05.003
1383-5718/

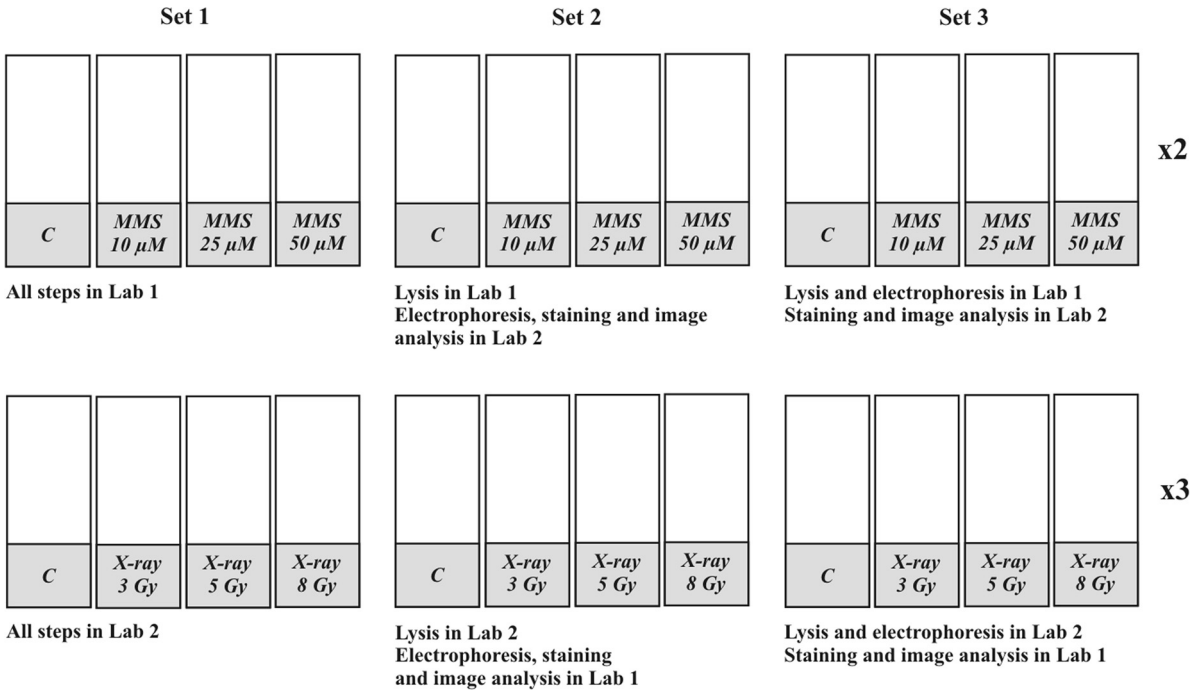


**Fig. 1.** Inter-laboratory comet assay designs. "C" is control, "x2" and "x3" are the numbers of parallel slides.

each laboratory. The specimens were divided into three Sets, four slides in each. Set 1 was a control group for which all procedures of the comet assay protocol were performed in the laboratory where slides were prepared. Set 2 was designed to reveal inter-laboratory variations in the results associated with the electrophoresis procedure. Set 3 was used to estimate how different slide staining procedures and software for the comet score affect the results.

### 2.1. Cells

Bone marrow cells from C57B1/6 mice were used. Animals were sacrificed by cervical dislocation. Epiphyses of the femurs were cut off; bone marrow cells were flushed with RPMI-1640 media containing 10% fetal calf serum (2 ml per bone).

#### 2.1.1. Experimental procedures performed in Lab 1

*2.1.1.1. MMS treatment.* Bone marrow cells were exposed to methyl methanesulfonate (MMS) at concentrations of 10, 25 and 50 μM, for 3 h at 37 °C.

*2.1.1.2. Alkaline comet assay.* After treatment with MMS, the cell suspension (50 μl) was mixed with 350 μl of LMA (1% in PBS, agarose Type IV EEO, Panreac, Spain, Cat. No. 374116.1206), dropped to slides pre-coated with 1% normal melting point agarose. Then the slides were covered with coverslips and placed on ice. After solidification of agarose (in about 10 min), the coverslips were carefully removed, the slides were placed in a glass cuvette (Schifferdecker type) filled with lysis solution 'A' (10 mM Tris–HCl, pH 10, 2.5 M NaCl, 100 mM EDTA-$Na_2$, 1% Triton X-100, 10% DMSO, 4 °C) and incubated for at least 1 h at 5–7 °C in the dark. Then the slides were placed in an electrophoresis chamber (SubCell GT, "Bio-Rad") with alkaline electrophoretic solution (300 mM NaOH, 1 mM EDTA-$Na_2$, pH > 13, 8 °C) and alkali treatment was carried out during 20 min. Electrophoresis was performed in the same solution for 20 min at an electric field strength (EFS) of 2.14 V/cm calculated as an applied voltage per unit length of the platform for slides (V/cm); the applied voltage was 32 V and the current was ~300 mA.

In some experiments, electrophoresis was accompanied by measuring the temperature of electrophoretic solution (at the top of the well near the anode) using an MS6501 digital thermometer (Precision Mastech Enterprise). All of these steps were conducted under dim light to prevent the occurrence of additional DNA damage.

After electrophoresis, the slides were fixed in 70% ethanol, dried at room temperature and stored until staining. Immediately prior to microscopic analysis, the slides were stained with SYBR Green I (1:10,000 in TE buffer, Invitrogen, USA) for 30 min in the dark. Analysis was performed on a Mikmed-2 12T epifluorescence microscope ('LOMO', Russia) combined with a high-resolution digital camera (VEC-335, Russia), at 200× magnification. The images of comets were analyzed by using CASP v.1.2.2 software [11]. At least 100 comets were analyzed per slide.

#### 2.1.2. The experimental procedures performed in Lab 2

*2.1.2.1. X-ray treatment.* To induce DNA strand breaks and alkali-labile sites, cells immobilized in LMA on glass slides were exposed to X-rays delivered by a RTD-250-15-1 X-ray unit (Russia) operated at 200 KV and 20 mA. Radiation was filtered through 1 mm Al and 1 mm Cu. The dose rate was 1.12 Gy/min. During irradiation, slides were placed on an ice cold surface.

*2.1.2.2. Alkaline comet assay.* Slides were prepared according to the procedure described previously [12]. Slides, after exposure to a DNA-damaging agent, were placed in the lysis solution 'A' (without 10% DMSO) and incubated for 24 h at 5–7 °C in the dark. In all experiments the alkaline denaturation lasted for 20 min and took place in a separate tray, i.e. outside the electrophoresis chamber. Alkaline denaturation and electrophoresis were carried out in a refrigerator. The electrophoretic solution and the chamber were preliminarily cooled to 4–6 °C. The electrophoresis was performed for 20 min at 2.0 V/cm, applied voltage 27 V. The temperature control of the upper layer (1–2 mm) of electrophoretic solution was performed continuously by using a Karmanov microthermistor. The measurements were carried out at a central point of the electrophoresis chamber. After electrophoresis, the slides were rinsed in distilled water and placed for 1 h in a solution of ethidium bromide (2.0 μg/ml) in PBS.

Before analysis, the slides were rinsed with distilled water for 5–10 min to remove unbound dye. Analysis of comet images was performed with a Lyumam-I-3 microscope ('LOMO', Russia). Digital images of comets were captured with a Nikon CoolPix 995 camera (Japan). For each slide, 50 images of comets were acquired. Image analysis was performed using the software package developed at the Institute of Cell Biophysics of RAS [13].

Characteristics of electrophoresis chambers used and some conditions of electrophoresis procedure are presented in Tables 1 and 2. In both laboratories, the percentage of DNA in the comet tail was used as an end-point of DNA damage (%TDNA).

### 2.2. Exchange of slides between Labs

Two groups of slides – after lysis (=Set 2) and after electrophoresis (=Set 3) (steps of the Comet assay protocol) were transported between Labs. The transportation time was 3–4 h. Slides after the lysis step were transported in a high-humidity chamber hermetically sealed to prevent drying of agarose and upon arrival were placed again in a cooled lysis solution. After the electrophoresis step, the slides were subjected to neutralization before being transferred to the other Lab, where they were stained and analyzed.

### 2.3. Infrared imaging

Measurements of temperature distribution in the superficial layer of the electrophoretic solution (SE-1 tank, Lab 2) during electrophoresis procedure were performed in the Laboratory of mechanisms of biostructure's organization, ITEB RAS, by means of a high resolution real-time infrared (IR) thermograph. A cooled type focal plane array infrared camera ('TITANIU', CEDIP, France) with sensitivity in 3–5 μm spectral window and 320 × 256 pixels spatial resolution was used. The