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TECHNICAL ASPECTS OF THE ELECTROPHORESIS STAGE IN THE COMET ASSAY

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❀ Inter- and intralaboratory variability of results is still a serious issue in the comet assay. There are several technical conditions of procedure, which may critically affect the results and electrophoresis terms were identified as main. A comparative assessment of the expected and actual electric field strength in five electrophoretic tanks and the contribution of the revealed differences to the variability in DNA damage carried out. Only for one tank, the measured electric field strength coincided with the expected 1 V/cm, while for four it ranged from 0.6 to 2.0 V/cm. The values of DNA damage assessed in the same samples of mouse kidney cells differed between tanks up to 4.7-fold for induced and up to 10-fold for spontaneous DNA damage. High local variations in the electric field strength and solution temperature across the platform as well as in %DNA in the tail of identical cell samples within electrophoresis runs also revealed. These variations were reduced by recirculation of electrophoresis solution. The results show that discrepancy between the estimated and the actual electric field strength can be reason of inter-laboratory variation of the comet assay results. Recirculation of the solution during electrophoresis will be useful to control of intra-laboratory and intra-assay variations.

❀ **Keywords:** comet assay; inter- and intralaboratory variability; DNA damage; electric field strength; voltage; electrophoresis tank.

ТЕХНИЧЕСКИЕ АСПЕКТЫ ПРОВЕДЕНИЯ ЭТАПА ЭЛЕКТРОФОРЕЗА В МЕТОДЕ ДНК-КОМЕТ

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❀ Причины меж- и внутрилабораторной вариабельности данных, полученных в исследованиях методом ДНК-комет, на сегодняшний день не до конца ясны. Гель-электрофорез является определяющим этапом при использовании метода анализа поврежденности ДНК единичных клеток. В настоящей работе проведена сравнительная оценка расчетной и фактической напряженности электрического поля в пяти различных электрофоретических камерах и вклада выявляемых различий в вариабельность оцениваемой поврежденности ДНК. Только для одной камеры измеренная напряженность совпала с расчетной — 1 В/см, тогда как для четырех варьировала от 0,6 до 2,0 В/см. Оцененные в одних и тех же образцах клеток почек мышей значения поврежденности ДНК различались между камерами до 4,7 крат для индуцированной и до 10 крат для спонтанной поврежденности ДНК. Выявлена высокая локальная вариация напряженности электрического поля и температуры раствора по площадке камер и вариабельность поврежденности ДНК в идентичных образцах клеток в условиях одного электрофореза. Таким образом, несоответствие при электрофорезе расчетной (теоретической) и фактической напряженности электрического поля может служить причиной межлабораторной вариабельности данных метода ДНК-комет. Рециркуляция раствора в ходе электрофореза позволяет значимо снизить внутриэкспериментальную и внутрилабораторную вариабельность результатов.

❀ **Ключевые слова:** метод ДНК-комет; повреждение ДНК; меж- и внутрилабораторная вариабельность; электрофоретическая камера; напряженность электрического поля; напряжение.

INTRODUCTION

Assessment of DNA damage as a biomarker in biomedical and genotoxicological studies *in vitro* and *in vivo* has become widespread following the introduction of single cell gel electrophoresis (SCGE, comet assay) [1, 2].

The important characteristics of this technique, especially when used as a robust test in genotoxicology,

are accuracy and reproducibility. The inter- and intra-laboratory variability of data obtained using comet assay has remained an unresolved setback, giving rise to uncertain or contradictory data [3, 4].

The European Comet Assay Validation Group (ECVAG) conducted a large-scale international comparison study aimed at determining the inter- and intra-laboratory precision and reproducibility of comet assay data [5, 6]. The result revealed a high inter-laboratory variability of data which, according to experts, was due to usage of different protocols of the technique. It was found that the critical factors that determined the outcome of the comet assay include the concentration of agarose gel, duration of alkaline denaturation/electrophoresis, and electric field strength [3, 4, 7]. Temperature of the solution during electrophoresis also has a significant effect on the estimated parameters [8, 9]. Standardization of experimental procedures in subsequent ECVAG studies reduced the intra-laboratory variability of results; however, the inter-laboratory variability remained high [10–12].

The use of various equipment by laboratories *a priory* does not allow for complete standardization of the protocol. Technical tools for obtaining and analyzing images of DNA comets do not have a significant effect on the results obtained; whereas, contribution of the variability of characteristics of equipment used for gel electrophoresis stage is not fully understood [13–15].

Traditionally, for the comet assay, electrophoretic chambers, which are designed for agarose gel electrophoresis of nucleic acids or chambers with a similar design specially designed for the technique, are used. In this case, strength of the electric field (as for gel electrophoresis of nucleic acids) is determined by ratio of the applied voltage to distance between the electrodes [15, 16]. There are repeatedly expressed opinions that this principle of calculating strength during alkaline electrophoresis in comet assay may be erroneous [4, 15, 17]. At the same time, only one study has assessed the correspondence between calculated and actual field strength of a single electrophoretic chamber [3].

This study aimed to perform a comparative estimate of calculated and actual electric field strengths in five different electrophoretic chambers, and contribution of the revealed differences to variability of the spontaneous and induced DNA damage.

MATERIALS AND METHODS

Preparing of DNA comet slides

This study was performed on mature male F_1 hybrid mice (CBA × C57Bl/6) (weight = 22–24 g, aged = 6–8 weeks) obtained from the Stolbovaya animal nursery. The experiments were conducted in accordance to the European Parliament and Council of European Union directives (2010/63/EU) on protection of animals used for scientific purposes. The study was approved by the Commission on Biomedical Ethics of the V.V. Zakusov Research Institute of Pharmacology (protocol No: 6 of 04.07.19). In order to comply with the 3R principles, tissue samples from animals of positive and negative controls in the preclinical drug studies were used.

For induction of DNA damage, 30 mg/kg of methyl-methane sulphonate (MMS, Sigma Aldrich) was intraperitoneally administered to the animals. Equivalent volumes of saline were intraperitoneally administered to the animals used as negative control. Three hours after the saline or MMS administration, the animals were withdrawn from the experiment by decapitation. In the first series of experiments, DNA damage was assessed in kidney cells, while kidney and bone marrow cells were used for the second series of experiments.

Femoral bones were isolated, epiphyses were excised, and bone marrow cells were flushed with 2 ml of pre-cooled at 6 °C phosphate saline buffer (containing 20 mM $EDTANa_2$ and 10% dimethyl sulfoxide (pH 7.4)). Kidneys were minced (with a glass rod) in a glass tube containing 3 ml of the same buffer and left for 5 min at room temperature to precipitate large tissue fragments.

For each experiment, identical slides from one cell suspension were prepared. In brief, a cell suspension of 60 μL was placed into tubes containing 240 μL of 1% solution of low-melting agarose in phosphate saline buffer heated to 42 °C (Termit microthermostat, Russia) and resuspended. Several of such test tubes were prepared. 35 μL of agarose solution with cells was applied onto glass slides precoated with 1% universal agarose, covered with glass covers (24 × 24 mm), and placed on ice. The glass covers were carefully removed and microscope slides were placed in a glass cuvette (Schiffendecker type) with lysis buffer (10 mM TrisHCl [pH 10], 2.5 M NaCl, 100 mM EDTA-Na_2, 1% Triton X-100,

10% dimethyl sulfoxide) (precooled to 6 °C) and incubated for 1 hour [22]. At end of lysis, the slides were placed in cuvettes containing a buffer solution (300 mM NaOH, 1 mM EDTA-Na_2, pH > 13) for electrophoresis (precooled to 6 °C) and incubated for 20 min to denature DNA and realize alkali-labile sites.

Electrophoresis

Electrophoresis was performed in electrophoretic chambers SE-2 (Helikon, Russia), Sub-Cell 192 (BioRad), multiSUB Screen 32, CSL–COM40, and COMPAC-50 (Cleaver Scientific) in electrophoresis solution (precooled to 6 °C) for 20 min at an initial current of 300 mA (source Power PRO 300, Cleaver Scientific). In CSL–COM40, Sub-Cell 192, and multiSUB Screen 32 chambers, slides containing cells were placed on 12 sections of the chamber platform as illustrated in Fig. 1. The remaining space on the platform was filled with cell-free agarose slides to cover gaps between the slides. 8 slides containing cells which completely filled the chamber area were placed in chamber SE-2.

The applied voltage (V) was equal to distance (cm) between the chamber electrodes – the calculated electric field strength was 1 V/cm. The actual electric field strength was determined by measuring of the electric potential in electrophoresis solution at a height of ~1 mm above the slides (Mastech MAS838 multimeter with gold-plated probes) at the beginning (minute 0–1) and end (minute 19–20) of electrophoresis.

In the first series of experiments, actual electric field strength was determined by measuring the electric potential between two points in the middle part of the chamber platform located at a distance of 10 cm. For the COMPAC-50 chamber, electric potential was measured between two points at a distance of 7.6 cm (along the slide width) in left and right parts of the chamber by immersing probes in the electrophoresis solution to a depth of 1 mm near the slides surface. In the second series of experiments, for assessment of the local electric field strength, electric potential was measured in a centimeter segment above the slides with cells (Fig. 1). Temperature of the electrophoresis solution was measured in the same segments in the second series of experiments using DT-886OB infrared digital thermometer (CEM). In all cases, average value for three measurements was calculated. In experiments with recirculation of electrophoresis solution, B3-V PER peristaltic pump (Etatron D.S.) with Santoprene hose (Santoprene®) resistant to alkaline solutions was used. In the course of electrophoresis, solution from cathode reservoir of the chamber was supplied to anode at a rate that yielded a change of 1.5 volumes of solution in the reservoirs (Fig. 1).

Analysis of preparations

After electrophoresis, the slides were washed in phosphate saline buffer, fixed in 70% ethanol for 15 min, allowed to dried, and stored at room temperature until analysis. Slides were stained with SYBR Green I fluorescent dye (Invitrogen, 1 : 10000 in TE buffer with 50% glycerol, pH 8.5) for 20 min. Digital images from slides (10–15 per slide) were obtained using epifluorescence microscope Mikmed-2 12T (Lomo, Russia) with high-resolution chamber (VEC-335, EVS, Russia) at a total magnification of 800 (optical × 20, digital × 40). On each slide, 100 DNA comets were analyzed and average value of the percentage tail DNA (DNA in the "tail", %) was calculated using CASP 1.2.2 software.

The hypothesis on equality of mean values of the DNA damage index for electrophoresis with or without recirculation was tested using Mann–Whitney *U*-test. Equality of general variances of the samples was tested using Fisher's *F* test

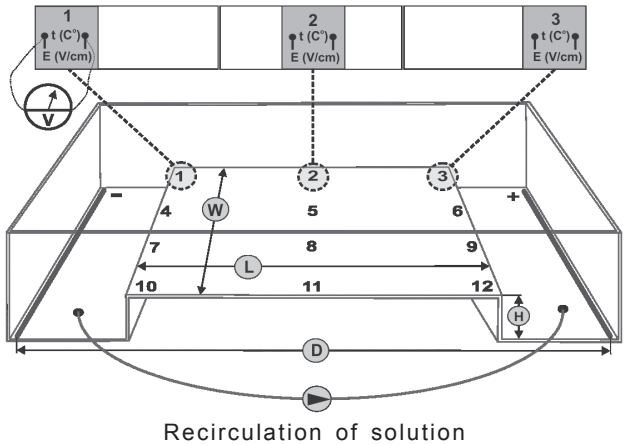

Fig. 1. Geometric parameters of chambers, location of slides with cells on the platform and sections (indicated by digits 1–12) for determining the local electric field strength and temperature of electrophoresis solution in the CSL–COM40, Sub-Cell 192, and multiSUB Screen 32 chambers. M – interelectrode distance; H – height, L – length, W – width of the chamber platform

RESULTS

The Table 1 presents the geometric characteristics of the chambers and actual values of the electric field strength measured during electrophoresis (the first series of experiments). For CSL–COM40 only, the measured value coincided with the calculated value. For SE-2 chamber, difference between the calculated and measured values was the smallest. The Sub-Cell 192 chamber is equipped with an additional stand installed on top of the platform, which enables it to increase the size of the agarose gel and, accordingly, the number of slides while using the chamber for the comet assay. While performing electrophoresis with an additional platform, the measured value of strength was 1.2 V/cm, whereas the strength increased to 2.0 V/cm without the additional platform.

In the multiSUB Screen 32 chamber with the voltage of 46 V and electrophoresis solution height of 2 mm above the glass, current was about 420 mA. It was technically impossible to reduce the current to the recommended 300 mA by removing some portion of the solution, since the solution did not cover the slides in this case. The measured strength value with the parameters specified was significantly higher than the calculated value (1.4 V/cm).

The COMPAC-50 chamber has a unique patented design with vertical orientation of DNA comet slides (Fig. 2), which is advantageous due to its compactness and large capacity (up to 50 slides). Measurements have shown that at the manufacturer's recommended voltage of 21 V, electric field strength near the surface of the slides is 0.6 V/cm.

Analysis of DNA comet preparations revealed a high variability in the levels of spontaneous and induced DNA damage assessed for the same sample of mouse kidney cells during electrophoresis in the different chambers (Table 1, Fig. 3). The highest levels of DNA damage were obtained using the Sub-Cell 192 chamber without additional platform (E = 2.0 V/cm), and the lowest were obtained using the COMPAC-50 chamber (E = 0.6 V/cm).

The supplied voltage was empirically selected for the chambers, such as that the electric field strength was 1 V/cm (Table 1) and the experiments with preparations of mouse kidney cells were repeated. As a result of unification of electric field strength in the chambers, similar levels of both spontaneous and induced DNA damage were obtained. The exception was the COMPAC-50 chamber, for which similar results were obtained during electrophoresis for 30 min (1 V/cm, 680 mA; see below).

Table 1

The dimensions of electrophoretic tanks (figure 1) and spontaneous and induced DNA damage in kidney cells of mice at electrophoresis with an expected and actual electric field strength 1 V/cm

Tank	Dimensions (cm)				Solution volume (ml) *	At the voltage applied according D			At E = 1 V/cm		
	W	H	L	D		E. V/cm **	% DNA in tail ($m \pm$ SD) ***		Voltage (V)	% DNA in tail ($m \pm$ SD)	
							Control	MMS 30 mg/kg		Control	MMS 30 mg/kg
SE-2	13	2.4	17.6	27	550	1.1	1.9 ± 0.9	13.1 ± 1.7	26	1.6 ± 0.8	12.2 ± 1.5
CSL–COM40	35.5	3.3	14.5	25	1900	1.0	3.4 ± 2.8	13.3 ± 3.7			
Sub-Cell 192 (w/o add. platform)	26	4.1	15.2	32	2080	2.0	9.0 ± 1.7	20.7 ± 1.9	24	1.7 ± 0.7	9.9 ± 0.9
Sub-Cell 192 (with add. platform)	26	4.6	25.7	32	2150	1.2	2.2 ± 0.3	15.5 ± 2.9	30	1.5 ± 0.4	12.8 ± 2.2
multiSUB Screen 32	27	2.5	32	46	1280	1.4	4.9 ± 1.9	16.7 ± 3.2	38	1.8 ± 0.5	11.8 ± 1.6
COMPAC-50	10.6	3.2#	18	21	550	0.6	0.9 ± 0.3	4.6 ± 0.6	28	1.5 ± 0.8##	12.9 ± 2.1##

Note. * Depth of electrophoresis solution above slides ~2 mm. ** Measurements at the beginning of electrophoresis at a current ~300 mA (except COMPAC-50 and Multi SUB Screen 32 tanks; see in text). *** For 12 slides (CSL-COM40, Sub-Cell 192 and Multi SUB Screen 32 tanks) and for 8 slides (COMPAC-50 and SE-2 tanks). # Depth of electrophoresis solution (fig. 2). ## Electrophoresis 30 min. W – width, L – length and H – height of platform of tanks; D – distance between electrodes (fig. 1).

Analysis of the data revealed a significant variation in the average DNA damage between identical slides obtained under the same electrophoresis conditions. The CSL–COM40 chamber recorded the highest percentage tail DNA both for spontaneous (range = 1.1–8.8%) and induced (range = 4.1–17.9%) DNA damage. It has been suggested that this data variability may be due to lack of homogeneities in field strength and/or temperature across the chamber platform. In the experiments described above, the field strength was determined by measuring the electric potential above the slides on a ten-centimeter segment in the middle part of the chamber platform. Furthermore, in the CSL–COM40 chamber, the strength was determined in 12 sections of the platform (Fig. 1) by measuring the potential difference in a section at one centimeter above the slides under standard conditions and with recirculation of the electrophoresis solution. At the same time, temperature of the electrophoresis solution was determined in these sections.

Measurements revealed pronounced differences in strength which varied from 0.8 to 1.5 V/cm at the start of electrophoresis and from 1.0 to 1.3 V/cm at the end (Table 2). In this case, the highest values were noted in the sections located near the cathode reservoir (sections 1, 4, 7, and 10), while the lowest were near the anode reservoir (sections 3, 6, 9, and 12). Temperature of the electrophoresis solution at the start of electrophoresis varied from 8.0 to 12.6 °C and increased significantly in all sections to similar values at the end.

Recirculation of the electrophoresis solution led to equalization of the electric field strength across the chamber area of 0.9–1.1 V/cm. Temperature of the electrophoresis solution under recirculation conditions at the start of electrophoresis also differed between the sections; however, it did not increase at the end (though decreased in some sections).

Similar measurements were conducted in the Sub-Cell 192 and multiSUB Screen 32 chambers during electrophoresis of DNA comet preparations of the bone marrow and kidney cells from intact mice and those treated with 30 mg/kg of MMS (second series of experiments).

In the Sub-Cell 192 chamber, without recirculation of electrophoresis solution, the strength values varied from 0.8–1.1 V/cm (Table 3). Under conditions of recirculation, the strength in all sections (except for section 11) was 1.0 V/cm at the start of electrophoresis and increased to 1.2 V/cm at the end. The mean values of DNA damage did not differ significantly for electrophoresis with and without recirculation. Also, a high level of variability was revealed between identical slides of intact bone marrow cells during electrophoresis without solution recirculation, with the range of 0.9 to 3.6% tail DNA (CV = 41.9%). Under conditions of electrophoresis with solution recirculation, the coefficient of variation decreased to 14.3%.

A lower scatter of values was revealed in the analysis of DNA comet slides of bone marrow cells of

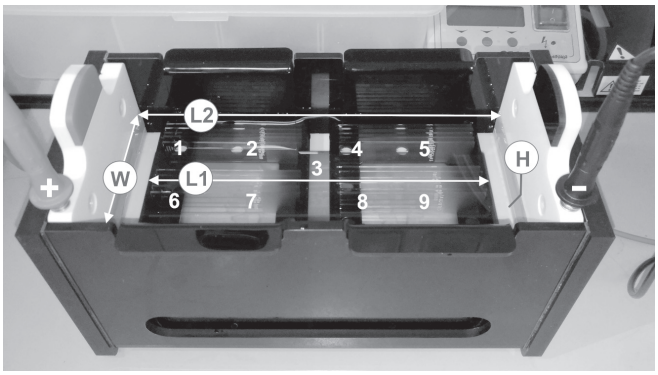

Fig. 2. COMPAC-50 chamber with vertical orientation of slides. 1–9 sections for determining the electric field strength. M – interelectrode distance; H – height of the solution for electrophoresis, L – length, W – width of the chamber reservoir

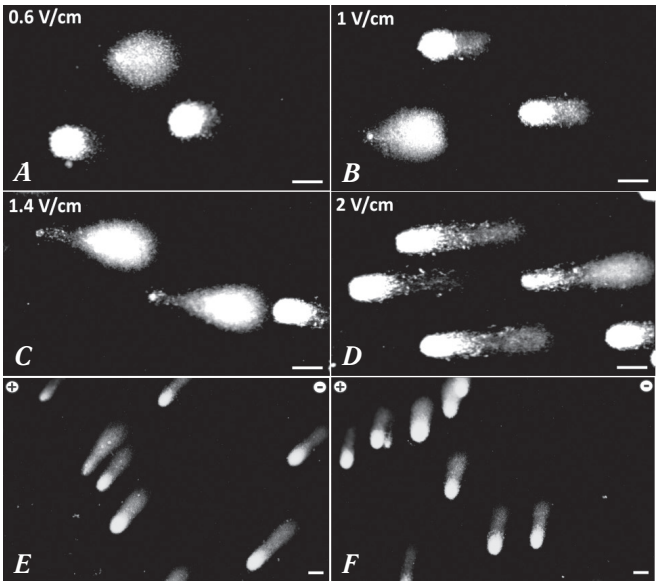

Fig. 3. DNA comets' morphology at different electric field strength (*A*, *B*, *C*, *D*; electrophoresis 20 minutes) and at 2-times increased of recirculation speed (*E*, *F*; Multi SUB Screen 32 tank). Bar scale – 50 μm

Table 2

Values of the electric field strength and temperature of electrophoresis solution in 12 sites of CSL-COM40 tank

Number of measurement site	Without recirculation		With recirculation	
	t_s/t_f, °C *	E_s/E_f, (V/cm)	t_s/t_f, °C	E_s/E_f, (V/cm)
1	8.0/18.7	1.4/1.3	9.4/9.4	1.0/1.0
2	9.4/18.2	1.2/1.1	11.1/10.8	1.0/1.0
3	9.8/17.0	0.9/1.1	14.1/10.3	1.1/1.1
4	10.0/19.1	1.4/1.2	9.7/9.5	1.0/1.0
5	11.3/16.7	1.1/1.1	12.2/9.9	1.0/1.0
6	11.7/17.5	0.8/1.0	13.3/10.5	1.1/1.0
7	10.4/18.0	1.5/1.3	10.5/10.0	1.1/1.0
8	10.1/18.3	1.3/1.1	12.1/11.0	1.0/1.1
9	10.6/17.3	0.8/1.0	14.1/11.7	1.1/1.0
10	12.6/17.6	1.5/1.3	10.8/9.5	0.9/1.0
11	11.2/17.0	1.2/1.1	11.1/10.2	1.0/1.0
12	10.0/17.6	0.9/1.0	12.2/10.4	1.0/1.0
CV, %		22.6/10.2		6.1/3.8

Note. * here and in tables 3 and 4 – the values obtained at the beginning t_s; E_s (0–1 minutes) and at the end t_f; E_f (19–20 minutes) of electrophoresis

animals treated with MMS. The coefficient of variation in this case of electrophoresis was 8.6% without recirculation of solution and 4.6% under solution recirculation. As in the case of CSL–COM40 chamber without recirculation, temperature of the electrophoresis solution increased significantly at the end of electrophoresis but did not exceed 11.8 °C under recirculation. Moreover, in both cases, difference in temperature of solution above the slides and in the chamber tanks did not exceed 3 °C.

In the multiSUB Screen 32 chamber, the electric field strength across the platform varied within 0.8–1.2 V/cm at the start of electrophoresis and between 0.9–1.3 V/cm at the end (Table 4). At the same time, the picture opposite compared to the CSL–COM40 chamber was noted. Precisely, high values of strength were recorded in sections located near the anode reservoir, while low values were registered near the cathode reservoir. The recirculation of the electrophoresis solution somewhat reduced the unevenness of the electric field strength across the site.

Unlike the CSL–COM40 and Sub-Cell 192 chambers, solution recirculation neither prevented temperature variability across the chamber site nor increased the temperature at the end of electrophoresis. A twofold increase in recirculation rate did not affect the variability of strength and temperature (data not presented), but changed the direction of comets' tails on the slides. The analysis of such images was not performed (Fig. 3, *E* and *F*). The mean values of DNA damage did not differ significantly for electrophoresis with or without recirculation. DNA damage in intact kidney cells varied between slides from 0.7–2.8% of tail DNA ($CV = 36.1\%$). The coefficient of variation for slides with induced DNA damage in kidney cells was higher when compared to that of the Sub-Cell chamber ($CV = 16.2\%$). A decrease in coefficients of

Table 3

Values of the electric field strength and temperature of electrophoresis solution in 12 sites of Sub-Cell 192 tank (with additional platform) and correspond spontaneous and induced DNA damage in bone marrow cells of mice

Number of measurement site	Without recirculation				With recirculation			
	t_s/t_f, °C	E_s/E_f, (V/cm)	% DNA in tail		t_s/t_f, °C	E_s/E_f, (V/cm)	% DNA in tail	
			Control	MMS 30 mg/kg			Control	MMS 30 mg/kg
1	8.6/17.0	1.0/1.0	0.9	7.3	6.5/10.1	1.0/1.2	1.4	6.3
2	8.5/17.2	1.0/0.9	1.4	6.2	8.0/10.9	1.0/1.2	1.3	6.0
3	8.5/17.2	1.1/0.9	2.9	7.5	8.6/11.3	1.0/1.2	1.7	6.9
4	9.9/16.1	1.0/1.0	1.3	6.8	7.3/10.8	1.0/1.2	1.5	6.6
5	8.8/19.5	1.0/0.9	1.8	6.8	9.5/11.5	1.0/1.2	1.3	6.5
6	8.3/18.8	1.0/0.9	2.0	6.8	9.3/11.6	1.0/1.2	1.2	6.5
7	9.2/18.5	1.0/0.8	2.0	6.1	9.2/10.4	1.0/1.2	1.4	7.0
8	9.2/19.1	0.9/0.8	1.7	6.6	9.0/11.1	1.0/1.2	1.4	6.1
9	6.6/18.5	1.0/0.9	1.3	5.5	9.4/11.4	1.0/1.2	1.8	6.5
10	9.6/19.3	0.9/0.8	3.6	6.0	7.7/10.2	1.0/1.2	1.2	6.3
11	8.7/19.4	0.8/0.8	1.7	6.9	8.3/11.2	0.9/1.1	1.7	6.5
12	6.1/14.7	0.8/0.8	1.2	6.5	9.4/11.8	1.0/1.2	1.7	6.2
$m \pm SD$			1.8 ± 0.5	6.6 ± 0.4			1.5 ± 0.2*&	6.5 ± 0.3#&
CV, %		9.4/8.6	41.9	8.6		2.9/2.4	14.3	4.6

Note. * $p = 0{,}0002$; # $p = 0{,}04$ (*F*-test); & $p > 0{,}05$ (Mann–Whitney *U*-test) as compared with electrophoresis without recirculation.

Table 4

Values of the electric field strength and temperature of electrophoresis solution in 12 sites of MultiSUB Screen 32 tank and correspond spontaneous and induced DNA damage in kidney cells of mice

Number of measurement site	Without recirculation				With recirculation			
	t_s/t_f, °C	E_s/E_f, (V/cm)	% DNA in tail		t_s/t_f, °C	E_s/E_f, (V/cm)	% DNA in tail	
			Control	MMS 30 mg/kg			Control	MMS 30 mg/kg
1	8.9/20.6	0.8/1.0	1.9	9.6	8.3/13.7	1.0/1.0	1.3	9.2
2	9.2/20.8	0.8/0.9	1.2	10.4	8.7/13.5	0.9/0.9	1.4	11.1
3	9.0/15.8	1.2/1.2	2.3	9.9	8.6/14.2	1.2/1.0	1.7	13.5
4	7.9/18.8	0.8/1.0	2.6	13.3	9.3/12.1	1.0/1.0	1.8	12.2
5	9.3/20.5	0.8/0.9	1.1	13.8	8.8/14.8	0.9/0.9	0.9	10.3
6	8.8/10.6	1.2/1.2	2.1	14.7	7.0/12.1	1.2/0.9	2.3	12.0
7	9.1/18.7	0.9/1.0	1.9	15.1	8.8/13.4	1.0/1.0	1.6	13.1
8	8.7/20.4	0.9/0.9	1.4	11.4	8.0/14.3	0.9/1.0	1.1	8.8
9	8.1/15.2	1.2/1.3	0.7	10.4	7.8/17.7	1.1/0.8	2.1	10.4
10	8.9/21.7	0.9/1.0	2.1	11.5	6.2/13.3	1.0/1.0	2.1	10.8
11	9.0/21.5	0.9/0.9	1.3	10.8	7.8/17.8	0.9/0.9	1.7	8.7
12	8.5/15.0	1.0/1.1	2.8	10.8	7.7/16.8	1.0/0.8	1.8	12.1
$m \pm SD$			1.8 ± 0.5	11.8 ± 1.6			1.7±0.4*&	11.0±1.3*&
CV, %		17.1/13.3	36.1	16.2		10.8/8.3	25.4	14.6

Note. * $p > 0{,}05$ (*F*-test); & $p > 0{,}05$ (Mann–Whitney *U*-test) as compared with electrophoresis without recirculation.

variation in the indices of spontaneous and induced DNA damage was recorded as compared to electrophoresis without solution recirculation; however, it was not as pronounced as in the case of Sub-Cell 192 chamber.

The special aspects of electrophoresis in the COMPAC-50 chamber are worth noting. At the manufacturer's recommended voltage (21 V), the current and field strength was 420 mA and 0.6 V/cm, respectively. An electric field strength of 1 V/cm was achieved with an applied voltage of 28 V; however, the current increased to 680 mA, which is within the limit of the power source. Experiments with the measurement of local strength at 9 sections (Fig. 2) revealed that at the start of electrophoresis, the strength was 1.0 V/cm in all sections (1.2 V/cm in section 3 between the slides). By the end of electrophoresis, an increase in the current strength up to the limit of 700 mA led to a drop in voltage output of the power source down to 25 V (transition from voltage stabilization to current stabilization) and, accordingly, a drop in the field strength and its variation in the sections from 0.7 to 0.9 V/cm.

DISCUSSION

In horizontal agarose gel electrophoresis of nucleic acids, the ratio of applied voltage to distance between the electrodes is used to calculate the electric field strength regardless of the chamber geometry [15, 16]. Since in the comet assay use electrophoretic chambers of the same design, this principle was adopted by default when calculating the recommended voltage of 1.0 V/cm, when the applied voltage (V) is equal to the interelectrode distance (cm). Among the chambers used in our study, only one of them had a strength measurement value that coincided with the calculated value, while the four others had a strength measurement that varied from 0.6 to 2.0 V/cm. As a result, the DNA damage values estimated in the same sample of mouse kidney cells differed between chambers by up to 4.7 times when assessing the MMS-induced DNA damage and up to 10 times when assessing the spontaneous DNA damage. The discrepancy between the measured values of the electric field strength and the calculated values was reported in a study by Azqueta et al. [3]. Values below the calculated ones were observed, with the difference being inversely proportional to the applied voltage (electrophoretic chamber model was not specified). In our study, a measured electric strength lower than the calculated value was revealed for COMPAC-50 chamber, which has a non-standard configuration with vertical orientation of slides; however, in the other chambers, the values were higher.

To obtain optimal comet images, Olive et al. [17], perform electrophoresis for 20–25 min at a voltage equal to product of the distance between the chamber electrodes by 0.6. Such calculations related to all chambers used in this study gave voltage values significantly lower than those determined experimentally (1 V/cm) (Table 1). In a number of papers, it was proposed that strength should be calculated as ratio of the voltage supplied to length of the chamber platform (Fig. 1, *E*) [3]. The expected and actual values of strength with this principle of calculation coincide only for Sub-Cell 192 chamber without an additional platform and multiSUB Screen 32. In addition, with constant interelectrode distance and applied voltage, electric field strength on the platform with slides depends on its size. Thus, in the Sub-Cell 192 chamber, a voltage of 1 V/cm was achieved at an applied voltage of 24 V, whereas a voltage of 30 V was required when installing an additional platform to obtain the required field strength. These findings indicate that it is impossible to common the principle of calculating the electric field strength in the case of alkaline electrophoresis of DNA comets and in each case should be experimentally determine the applied voltage, providing the required field strength. A similar conclusion was made in a recent paper of Brunborg et al. [15].

Till date, there is no generally accepted rule on the value of electric field strength at which electrophoresis in comet assay should be performed. The OECD guidelines, referring to the results of studies by the Japanese Center for the Validation of Alternative Methods (JaCVAM), recommend electrophoresis for 20 min at a voltage of 0.7 V/cm [18]. According to a number of experts, electrophoresis is optimal at 1.15 V/cm for 20 minutes or at 0.7 V/cm for 30 minutes [3, 4, 15, 19]. According to literature, most researchers use a voltage of 1 V/cm, focusing on the recommendations issued by Tice et al. [20]. Our own long-term experience on the comet assay shows that electrophoresis at the strength of 1.0–1.2 V/cm

for 20 minutes is optimal. The use of lower values reduces the technique sensitivity, while higher values can lead to distorted analysis results. Detachment of DNA from the comet's "tail" and/or diffusion of small DNA fragments in the gel can lead to an underestimation of the DNA damage value (Fig. 3) [15].

It is believed that strength of the current does not affect DNA migration in the gel, but at the same time, in contrast to the electric field strength, a certain value is recommended for the current strength (300 mA) at the start of electrophoresis. It is not known for certain why exactly this current strength was initially accepted as optimal in the comet assay. There is an opinion that at time of the technique development, power supplies for electrophoretic chambers with a rated current load of more than 300 mA were not widely available due to their high cost [21]. The current strength depends directly on the height of the electrophoresis solution above the slides and is regulated by lowering/addition of the latter. Brunborg et al. [15] recommended electrophoresis with a solution height above the glasses at least 5 mm. According to the authors, this reduces the unevenness of temperature and strength across the chamber platform and, accordingly, the intra-experimental variability of the data. An insignificant decrease in the electric field strength with increase in current strength can be corrected by increasing the voltage applied to the chamber.

Along with the inter-laboratory variability, the intra-laboratory variability of the comet assay data represents an important problem [3, 4, 15]. Depending on the chamber used, coefficient of variation of DNA damage value for identical cell samples within the one electrophoresis run amounted to 8–16% and 36–42% induced and spontaneous DNA damage, respectively. Similar results were obtained in two studies [22, 23]. If for induced DNA damage, coefficient of variation did not exceed 14%, then for spontaneous DNA damage, it reached 52%. This fact is of particular importance when using the comet assay in research aimed at primarily assessing low levels of DNA damage such as genetic biomonitoring or clinical studies.

In the work Gutzkow et al. [19], where GelBond® minigels were used instead of standard slides, it was demonstrated that recirculation of electrophoresis solution reduces the coefficient of variation of DNA damage in irradiated cells from 26 to 7%. The authors suggested that the intra-experimental variability may be associated with heterogeneity of the electric field strength over the chamber platform. In a subsequent study, they showed that the field strength varies across the chamber platform ($CV = 10.5\%$) and solution recirculation, depending on speed, and that the coefficient of variation reduced to 0.5% [15]. For the three chambers in our study, the variability of the field strength (CV from 9.4 to 22.6%) was revealed. For the Sub-Cell 192 and CSL–COM40 chambers, recirculation of solution during electrophoresis significantly reduced the strength unevenness; however, this decrease was less pronounced in the multiSUB Screen 32 chamber. In the Sub-Cell 192 chamber, under recirculation conditions, the decrease in strength heterogeneity was accompanied by a decrease in the variability of spontaneous and induced DNA damage. In the multiSUB Screen 32 chamber, the variability of indices in both cases remained high. The lack of homogeneity of the field strength is presumably associated with local changes in the course of electrophoresis (especially in the specific electrical conductivity of the solution) due to temperature and/or ion concentration gradients in the chamber [15, 24]. It was revealed that an increase in temperature of the electrophoresis solution during electrophoresis is accounts for heterogeneity in the chamber, with higher values in the middle part of the platform (chamber SE-1) [9]. For the multiSUB Screen 32 chamber, in sections near the anode reservoir (3, 6, 9, 12), where the solution temperature was lower, higher strength values were registered. Experiments have shown that if the temperature of solution above the slides increases significantly by the end of electrophoresis, it increases by no more than 5 °C in the reservoirs of the chamber, thus forming temperature gradients. Recirculation leads to an active change of solution above the slides, contributing to equalization of temperature throughout the chamber (difference <3 °C). Probably, in a similar way, recirculation also prevents the emergence of electrolyte ions concentration gradients [15, 24]. A significant difference between the multiSUB Screen 32 chamber and Sub-Cell 192 chamber is the smaller volume of electrophoresis solution used (1,280 ml versus 2,150 ml). It can be assumed that the smaller the volume of the electrophoresis solution with similar

dimensions of the chamber platforms, the lesser the stabilizing effect of recirculation. Changes in pH of solution near the electrodes resulting from the water electrolysis can also affect the local electric field strength [25]. At the same time, given the high concentration of electrolyte in the solution, the high value (pH > 13.0) and the relatively short electrophoresis time, the contribution of pH changes is considered as insignificant [14, 15].

CONCLUSION

Overall, data obtained in this study show that discrepancy between calculated value of electric field strength during electrophoresis and actual value justifies the inter-laboratory variability of the comet assay results. Before conducting the experiments for a particular electrophoretic chamber, the voltage that provides the required values of the electric field strength and current strength should be determined empirically. To reduce intra-laboratory variability of data, especially when using large chambers, recirculation of the solution during electrophoresis is recommended, which stabilizes the temperature as well as electrochemical processes in the chamber. In combination with unification of other experimental conditions (concentration of agarose gel, duration of alkaline denaturation and electrophoresis), this will ensure inter- and intra-laboratory precision and reproducibility of the comet assay data.

The authors declare no conflicts of interest and no commercial implications in the planning, execution, and preparation for publication of this work.

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