Effect of Slide Positioning in Electrophoresis Chamber over Comet Assay Results

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1 ABSTRACT

Introduction: The single cell gel electrophoresis (aka comet assay), is commonly used in the regulatory battery of genotoxicity testing for different industries. Therefore it is important to determine which parameters can influence the variability of the results and therefore can potentially impact the conclusion.

Objective: Due to the voltage gradient, slides positioned in different sectors of the electrophoresis chamber could be subjected to different voltage. The present study was performed to evaluate how the position of the slide in the chamber impacted the median % tail intensity (% Tail DNA).

Material and Method: Slides were prepared using lung and duodenum cells from male Sprague Dawley rats. The negative control animals were untreated or dosed with water. The positive control animals were dosed with ethylmethanesulfonate (EMS). The route of administration was oral gavage. The electrophoresis chamber area was divided in two sides (left, right) and three sections (top, middle and bottom). Slides of the negative and positive control animal were placed in each side or section of the chamber. The average % Tail DNA was determined for each slide and the average % Tail DNA of all slides (total) for each animal was used as a standard for comparison between sections. To simulate the variability of a typical study design, permutations of the sides and sections were used to determine the minimum (min) and maximum (max) values, and the average deviation of the min and max values to the standard.

Results: A substantial difference was observed in the average % Tail DNA for the negative control slides positioned in different sections/sides of electrophoresis chamber. The percent deviation to the standard was between -34.9% and 30.5% for the lung and between -35.8% and 21.7% for the duodenum. For the positive control animal, the deviation to the standard was within ±10% for all areas for the lung and within ±3% for the duodenum. To determine if distributing the slides of a group through the chamber would suffice to minimize deviations, permutations with mixed regions were performed. The mixed groups of 3 slides consisted of one slide from each section and from one opposing side. The average deviation of the group to the standard was 1.7% (lung) and 2.1% (duodenum) for the negative control, and -3.6% (lung) and -1.5% (duodenum) for the positive control.

Conclusion: The variability of the results between positive control slides was sufficiently low to not impact the determination of a positive result. However, the variability of the negative control results (±35%) may deteriorate the confidence of negative or equivocal response. Ensuring the heterogeneous distributions of slides in the electrophoresis chamber from the same treatment group was sufficient to reduce the deviation to acceptable levels and ensure high confidence in the determination of negative responses.

2 INTRODUCTION

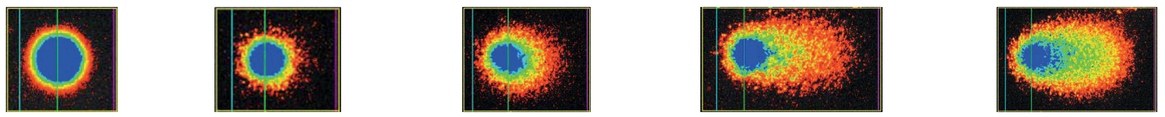
The comet assay is now well known and commonly used in the regulatory battery of genotoxicity testing, as well as in human biomonitoring, ecogenotoxicology, and basic research into the mechanisms of DNA damage and repair. Different parameters can highly influence the variability of the results and it is a serious issue. Agarose concentration, duration of alkaline incubation, and electrophoresis conditions (time, temperature, and voltage gradient) are particularly important. However, the voltage variability within the electrophoresis chamber is often omitted from consideration. The voltage gradient should be measured over the platform on which the slides are placed rather than simply between the electrodes, since that is where the electric potential pulls out damaged DNA from the nucleoids. Between the electrode and platform edge, in standard chambers, there is a relatively deep trough of electrophoresis solution, with low resistance, so that the voltage drop is much lower than over the platform where there is a thin layer of solution over the slides (sometimes as little as a few mm above the slides) and the resistance greatest; hence, measuring the total applied voltage and dividing it by the distance between the electrodes could gives an erroneous V/cm value. The purpose of the present study was to examine how the same sample can be affected by the position of the slide in the electrophoresis chamber.

3 MATERIAL AND METHOD

Samples preparation: Male Sprague Dawley Crl:CD(SD) rats were approximately 7 weeks old and weighed approximately 170 grams at initiation of dosing. They were acclimated and supplied food and tap water ad libitum. The negative control animals were untreated (duodenum) or dosed with water (lung). The positive control animals were dosed by intragastric gavage with two doses, separated by 21 hours, of ethylmethanesulfonate (EMS) at 200 mg/kg/day (10 mL/kg). There was 1 animal per condition. Three hours after the last administration, the animals underwent exsanguination from the abdominal aorta following isoflurane anesthesia. For this test, lung and duodenum tissues were used. For the lung, a cube of tissue of approximately 1 cm per side was manually disaggregated to prepare the single cell suspension samples. For the duodenum, cell suspensions were generated by gently scraping the surface of the gastric mucosa. Each cell suspension was mixed with 0.5% low melt agarose (keeping the % of low melt agarose at more than 90%). Aliquots of this cell/agarose mix were dispensed onto pre-coated (with 1.0% normal melt agarose) slides. Several slides per animal/tissue were prepared. Following solidification of the samples on slides, the slides were immersed in complete lysis solution (100 mM EDTA tetrasodium, 2.5 M NaCl and 10 mM Tris hydroxymethyl aminomethane with 1% v/v Triton X-100 and 10% v/v dimethyl sulfoxide), in a light proof box overnight at refrigerated conditions.

Electrophoresis: The slides were rinsed in purified water to remove excess salts from the slides. They were then transferred to a dry, level platform of a horizontal electrophoresis chamber (Cleaver Scientific Ltd). The slides from negative or positive control animal were positioned throughout the electrophoresis chamber (see Figure 1). Alkaline buffer (300 mM NaOH and 1 mM EDTA disodium, pH > 13.0) was added to the electrophoresis chamber in order to completely cover the slides by 2-3 mm. The DNA on slides was allowed to unwind in the alkaline buffer for 20 minutes. Then, a constant voltage of 16 V (with current at approximately 420 mA) was applied across the chamber (measured at approximately 0.63 V/cm) for 30 minutes. The temperature of the chamber was kept to below 10 degree celcius by use of a recirculating chiller. When the electrophoresis was completed, the slides were washed 3 times, for 5 minutes with neutralisation buffer (0.4 M Tris hydroxymethyl aminomethane, pH 7.5) and placed in chilled absolute ethanol for 30 minutes. The slides were then allowed to air dry and stored at room temperature in humidity controlled (with desiccant) lightproof boxes pending slide analysis.

Slides Analysis: The slides were wet-mounted with 45 µL of propidium iodide (20 µg/mL) and the cells visualized using a fluorescence microscope. The computerized image analysis Comet Assay IV™ from Perceptive Instruments was used for the analysis. 75 cells (lung) or 150 cells (duodenum) per slide were analyzed. The median % tail intensity (% Tail DNA), defined as the intensity of the comet tail relative to the total intensity (head plus tail), which reflects the amount of DNA breakage, was reported.



4 RESULTS

FIGURE 1
ELECTHROPHORESIS CHAMBER

			top
Black (left)	**1**	**1**	**Red (right)**
	2	**2**	
	3	**3**	
	4	**4**	
	5	**5**	
	6	**6**	
	7	**7**	
	8	**8**	
	9	**9**	
	10	**10**	
	11	**11**	
	12	**12**	middle
	13	**13**	
	14	**14**	
	15	**15**	
	16	**16**	
	17	**17**	
	18	**18**	
	19	**19**	
	20	**20**	
	21	**21**	
	22	**22**	
			bottom

TABLE 1
NEGATIVE CONTROL RESULTS

	Lung				Duodenum	
% Tail DNA	Average	Min	Max	Deviation[c]	Average	Deviation[c]
Total	0.71	-	-	-	1.27	-
left	0.94	0.67	1.17	30.5%	1.28	0.4%
right	0.50	0.46	0.55	-28.7%	1.26	-0.4%
top[a]	0.88	0.58	1.11	19.9%	0.88	-35.8%
middle[a]	0.74	0.65	0.81	3.8%	1.46	21.7%
bottom[a]	0.46	0.44	0.48	-34.9%	1.47	15.6%
mix[b]	-	0.56	0.88	1.7%	-	2.1%

a Slide position in the electrophoresis chamber: top from 1 to 7, mid from 8 to 15, bottom from 16 to 22
b The results obtained from 3 slides in each section of the electrophoresis tank (top, mid, bottom) were combined, with one (or two) slide(s) on the left side and two (or one) slide(s) on the right side.
c Average deviation in percentage between total average value to minimum and maximum values.
- Not applicable

TABLE 2
POSITIVE CONTROL RESULTS

	Lung				Duodenum	
% Tail DNA	Average	Min	Max	Deviation[c]	Average	Deviation[c]
Total	22.1	-		-	22.2	-
left	21.4	19.0	23.9	-3.8%	21.8	-1.7%
right	23.0	20.4	25.5	3.7%	22.6	1.7%
top[a]	23.6	21.6	26.2	8.0%	21.8	-1.1%
middle[a]	22.4	22.0	23.1	1.8%	22.0	-3.1%
bottom[a]	20.0	19.6	20.5	-9.5%	22.7	2.9%
mix[b]	-	19.1	23.6	-3.6%	-	-1.5%

a Slide position in the electrophoresis chamber: top from 1 to 7, mid from 8 to 15, bottom from 16 to 22
b The results obtained from 3 slides in each section of the electrophoresis tank (top, mid, bottom) were combined, with one (or two) slide(s) on the left side and two (or one) slide(s) on the right side.
c Average deviation in percentage between total average value to minimum and maximum values.
- Not applicable

5 DISCUSSION & CONCLUSION

The results show that significant variability is possible within an electrophoresis chamber. Each laboratory should therefore verify this type of assay variability for each of their electrophoresis chambers and adjust their setup in order to reduce this variable effect. An easy way to reduce the impact of this variability, it is to ensure the replicate slides of the same treatment group are distributed throughout the electrophoresis chamber. Therefore, the deviation will be reduced to acceptable levels.

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