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Searching of Standard Comet Assay Parameters for Detecting Lymphocyte DNA Damages Using Fourteen Different Test Conditions

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ABSTRACT

The alkaline single cell gel electrophoresis technique (SCGE), also known as the comet assay, is an evaluation method for estimating DNA damage and genotoxicity. This assay is used to measure DNA single-strand breaks, alkali-labile sites and DNA cross-linking in individual cells. Although the comet assay is a simple, sensitive, and rapid method to compare the results of previous studies, it is often difficult to interpret because of the variability among protocols. Standardisation and validation of the comet assay could provide invaluable information for the identification of hazardous substances and the risk assessment of environmental and occupational exposure. Further use of a standardised comet assay could include monitoring the effectiveness of medical treatment and the investigation of individual variation in response to DNA damage that may reflect genetic or environmental influences. In this study, the comet assay was evaluated for 4 parameters, including the tail moment (TM), tail intensity (TI), olive tail moment (OTM) and DNA tail (DNAt) length. 6 blood samples were collected from genetically unrelated healthy volunteers, and 7 different blood volume ranges (2.5 ml to 2.5 µl, diluted with PBS or RPMI) were tested for these parameters. The intraclass correlation coefficients (ICCs) were statistically significant among all 14 different test conditions ($r=0.87$, $p<0.001$ for TM; $r=0.86$, $p<0.001$ for TI; $r=0.73$, $p<0.05$ for OTM and $r=0.87$, $p<0.001$ for DNAt). In conclusion, the determination of DNA damage using the comet assay with small blood sample volumes can contribute worthwhile knowledge to future studies.

Keywords: Comet assay; DNA damage; validation; parameters.

Academic Discipline And Sub-Disciplines

Genetic Toxicology

SUBJECT CLASSIFICATION

DNA Damage Classification

TYPE (METHOD/APPROACH)

Validation of Comet Assay

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INTRODUCTION

The comet assay (single-cell gel electrophoresis technique; SCGE) is a simple, sensitive, fast and effective method for analysing small cell samples from any organ of eukaryotic organisms. This assay can be used to estimate DNA damage at the individual cell level by identifying single- and double-strand DNA breaks, DNA-DNA/DNA-protein cross-links, oxidative base damage, alkali-labile sites, and open repair sites(1-5). Rydberg and Johanson performed the first quantification of DNA damage in single cells embedded in agarose on microscope slides and lysed under mild alkali conditions(6). Staining of the nucleoid with acridine orange indicated a higher ratio of red to green fluorescence in cells with DNA damage. Ostling and Johanson first introduced micro gel electrophoresis technique under neutral conditions for increase sensitivity of detection DNA damage(7). Singh et al. (1) and Olive et al.(8) independently modified the assay by developing alkaline versions (pH>3 and pH =12.3, respectively). The Singh et al. (1) version has been the protocol of choice in biomonitoring studies. After the introduction of the alkaline modification, the breadth of applications and the number of studies using this assay have significantly increased.

The comet assay has been widely used for studies in genetic toxicology, clinical and radiation biology and DNA repair. Within the last decade, there has been an increasing interest in the comet assay due to the application of this method to environmental genotoxicity and biomonitoring studies. Human biomonitoring studies using the comet assay provide an efficient tool for measuring human exposure to genotoxicants, thus helping to determine risk (9). Currently, DNA damage detected by the comet assay may be considered to be an early indicator of genetic disease or cancer risk, an indicator of exposure to a wide variety of genotoxic agents, and a sensitive endpoint for detecting DNA damage(10-14).

The comet assay measures the electrophoretic migration of relaxed or fragmented DNA in relation to the nuclei of cells immobilised in an agarose gel. The distance and/or amount of DNA migration from individual nuclei are indicative of the number of strand breaks. In its simplest form, the comet assay only needs a few steps. First, cells are embedded in agarose on a microscope slide. The cells and nuclear membranes are then lysed in the agarose by detergent and high salt, and the DNA is subjected to alkaline electrophoresis. After staining cells with an appropriate dye, cellular DNA is visualised using a fluorescence microscope(1,2,15).

Currently, there is no standard protocol for the comet assay aside from the aforementioned basic steps. Different laboratories have modified some steps to meet their particular needs using several different parameters of DNA damage, such as tail intensity, tail length and/or tail moment. Because biomonitoring studies are observational methods rather than experimental studies, the comet assay has specific problems different from other standard genotoxicity tests. Consequently, the various protocol modifications and many experimental variables influence outcomes for the comet assay, and direct quantitative comparison among comet assay modifications is problematic(16,17). Validation is the process by which the reliability and accuracy of a method are established for a specific purpose (16,18). The comet assay requires validation and standardisation to avoid drawing incorrect conclusions. Once the comet assay is standardised and validated, it can provide invaluable information regarding the link between various exposures to environmental insults and disease(19,20).

Variability in the comet assay parameters and protocols among different laboratories erodes confidence in its results and makes comparison between the studies difficult. To obtain reliable results, the comet assay must be validated and standardised across all laboratories. Our aim in this study is to validate the comet assay technique by measuring 4 comet assay parameters using different amounts of RPMI, PBS, and blood samples.

MATERIALS AND METHODS

In this study, blood samples were obtained from 6 genetically unrelated healthy individuals (3 males and 3 females, mean age 28.50±6.12 years) with no occupational exposure to fuels or other chemicals. Each person was interviewed with a questionnaire covering a detailed medical, family and dietary history, including variables known to induce comet frequency. Exclusion criteria for subjects included the presence of illness, a history of radiotherapy and/or chemotherapy, malabsorption syndrome, and the use of therapeutic drugs known to be either genotoxins or reproductive toxins. Blood samples for each subject were collected between 7.30 and 8.30 a.m to minimise any confounding effects of oxidative stress. To avoid variation during analysis, all slides were analysed by the same investigator. This study design was approved by the institutional ethics committee (Approval number: 153-4853 in 2009).

Comet Assay

Lymphocytes were obtained from venous blood samples in anticoagulant heparin tubes. The comet assay was conducted under alkaline conditions with some modifications, as described by Singh et al.(1) In brief, conventional microscope slides were first covered with a layer of 0.5% normal agarose. Then, a 50-µl aliquot of the cell sample was mixed with 100 µl of 0.5% low melting point agarose and added to the slides, which were immediately covered with coverslips. After removing the coverslips, all slides were immersed in a 4 °C lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH was added to pH 10; 1% Triton X-100, 1% N-Lauryl Sarcosine, and 10% DMSO were freshly added) for 1 hour in the dark. The slides were placed in an electrophoresis tank and held for 20 minutes containing freshly prepared alkaline solution (300 mM NaOH, 1 mM EDTA, pH > 13), and the electrophoresis was performed for 20 minutes at 300 mA and 25 V. The slides were then taken from the tank and washed three times in neutralising buffer for 5 minutes (0.4 M Tris, pH=7.5). Afterwards, the slides were fixed in a series of ethanol for 5 minutes. Finally, the DNA was stained with ethidium bromide. Two slides were prepared for each sample, and 100 cells were randomly chosen and measured by a Comet Assay BAB Bs automatic image analysis system fitted with an Olympus BX50 fluorescence microscope. All results were evaluated in terms of 4 image analysis parameters: TM, OTM, TI, and DNAt.

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences, Windows version 16.0. The level of statistical significance was set at a p value of less than 0.05. Means and standard deviations were calculated for all data. Intraclass correlation coefficients (ICCs) were calculated to determine intra-examiner correlation (*r* value).

RESULTS

In this study, the comet method was validated for 4 comet assay parameters, TM, TI, OTM and DNAt, using 7 different blood volume ranges, from 2.5 ml to 2.5 µl, diluted with PBS or RPMI. Validation parameters of these 14 experimental conditions are shown in Table 1.

Table 1. The amount of blood and RPMI or PBS for 14 different test conditions.

TEST CONDITIONS	PBS	RPMI	AMOUNT OF BLOOD SAMPLES (µL)
1. CONDITION	2500 µL +8000 µL	-	2500 µL
2. CONDITION	-	2500 µL +8000 µL	2500 µL
3. CONDITION	1000 µL +3500 µL	-	1000 µL
4. CONDITION	-	1000 µL +3500 µL	1000 µL
5. CONDITION	1000 µL	-	100 µL
6. CONDITION	-	1000 µL	100 µL
7. CONDITION	500 µL	-	50 µL
8. CONDITION	-	500 µL	50 µL
9. CONDITION	100 µL	-	10 µL
10. CONDITION		100 µL	10 µL
11. CONDITION	50 µL	-	5 µL
12. CONDITION	-	50 µL	5 µL
13. CONDITION	25 µL	-	2.5 µL
14. CONDITION	-	25 µL	2.5 µL

According to the statistical analyses, highly significant ICCs were found among all 14 different test conditions ($p<0.01$). ICC results of the study were r=0.87 (0.63 to 0.98), $p<0.001$ for TM; r=0.86 (0.60 to 0.98), $p<0.001$ for TI; r=0.87 (0.63 to 0.98), $p<0.001$ for DNAt and r=0.73 (0.25 to 0.96), $p<0.05$ for OTM (Table 2). High ICC and $p<0.01$ values suggest that the results of the 14 different test conditions were consistent. Therefore, any of these 14 test conditions can be used for detecting DNA damage in terms of the TM, TI, DNAt and OTM comet parameters.

The ICCs of the 7 different comet assay conditions using various amounts of blood samples with PBS and RPMI were 0.596 and 0.811 for TM; 0.622 and 0.785 for TI; 0.598 and 0.800 for DNAt and 0.411 and 0.704 for OTM, respectively. These results show that the 7 different comet assay conditions with RPMI were more consistent than that of PBS, and test conditions with RPMI were statistically significant for all studied comet assay parameters ($p<0.01$ for TM, TI and DNAt; $p<0.05$ for OTM). However, only the TI parameter was statistically significant for PBS ($p<0.05$) (Table 3). The ICC between the two solutions was acceptable for the TM, TI, DNAt and OTM comet parameters (Figure 1).

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Table 2.The ICCs among the 14 different test conditions in 4 comet assay parameters (14 different test conditions were performed and two slides were analysed from each sample).

COMET PARAMETERS	Mean±S.D. N=6	r	95% CI	p
TM	3.89±0.94	0.867	0.631 – 0.978	<0.01*
TI	42367.85±11396.04	0.856	0.601 – 0.979	<0.01*
DNAt	85.74±27.32	0.866	0.630 – 0.978	<0.01*
OTM	653.36±730.40	0.728	0.246 – 0.955	<0.01*

* Statistically significant

Table 3. The ICC among 7 different conditions in various amounts of blood samples with PBS OR RPMI for 4 comet assay parameters.

COMET PARAMETERS	TEST CONDITIONS	Mean±S.D.	r	95% CI	p
TM	7 different conditions with PBS	3.92±0.96	0.596	-0.222 – 0.935	>0.05
	7 different conditions with RPMI	3.86±0.94	0.811	0.424 – 0.970	<0.01*
	7 different conditions with PBS and 7 different conditions with RPMI	3.92±0.96 and 3.86±0.94	0.782	0.595 – 0.883	<0.01*
TI	7 different conditions with PBS	42803.23±11262.39	0.622	0.143 – 0.939	<0.05*
	7 different conditions with RPMI	41932.46±11648.06	0.785	0.350 – 0.965	<0.01*
	7 different conditions with PBS and 7 different conditions with RPMI	42803.23±11262.39 and 41932.46±11648.06	0.773	0.578 – 0.878	<0.01*
DNAt	7 different conditions with PBS	85.91±27.72	0.598	0.217 – 0.935	>0.05
	7 different conditions with RPMI	85.59±27.25	0.800	0.396 – 0.968	<0.01*
	7 different conditions with PBS and 7 different conditions with RPMI	85.91±27.72 and 85.59±27.25	0.749	0.532 – 0.865	<0.01*
OTM	7 different conditions with PBS	581.15±540.85	0.411	0.055 – 0.905	>0.05
	7 different conditions with RPMI	725.57±881.36	0.704	0.105 – 0.953	<0.05*
	7 different conditions with PBS and 7 different conditions with RPMI	581.15±540.85 and 725.57±881.36	0.591	0.238 – 0.780	<0.05*

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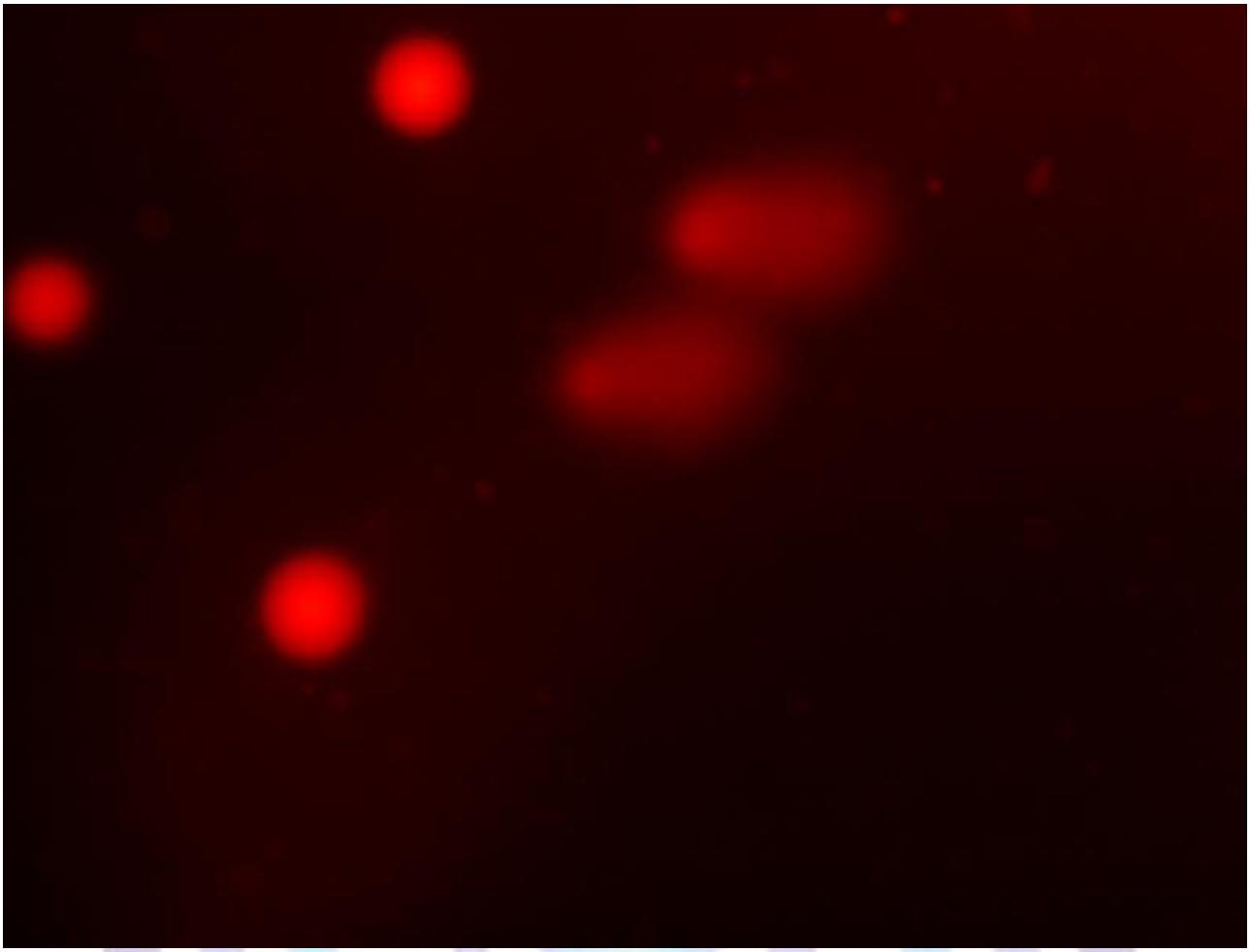

Figure 1: The comet assay analysis of blood samples

The ICCs between the two slides of the same sample and blood volume for PBS or RPMI in 4 comet assay parameters were statistically significant. The ICC values were r≥0.922 for RPMI and r≥0.807 for PBS in all comet assay parameters. Notably, there was excellent compatibility between the two slides using RPMI (Table 4). In all of the tables, the p<0.05 or p<0.01 values suggest that the associations among the test conditions were significant.

Table 4. The ICC between two slides in the same sample with the same blood volumes using either pbs or rpmi in 4 comet assay parameters.

COMET PARAMETERS	TEST CONDITIONS	r	95% CI	p
TM	with PBS condition	0.954	0.674 – 0.994	<0.01*
	with RPMI condition	0.999	0.995 – 1.000	<0.01*
TI	with PBS condition	0.871	0.079 – 0.982	<0.05*
	with RPMI condition	0.988	0.915 – 0.998	<0.01*
DNAt	with PBS condition	0.807	0.376 – 0.973	<0.05*
	with RPMI condition	0.975	0.818 – 0.996	<0.01*
OTM	with PBS condition	0.890	0.213 – 0.985	<0.05*
	with RPMI condition	0.922	0.439 – 0.989	<0.01*

* Statistically significant

DISCUSSION

The comet assay technique can be used to provide an early indicator for genetic disease or cancer and exposure to a wide variety of genotoxic agents; it is also a sensitive endpoint for detecting DNA damage (10,11,21). The comet assay is commonly used today because of its simplicity, sensitivity and rapidity. In spite of the advantageous features of this technique, the visual or automatic evaluation of samples, minor modifications in the protocol and the use of different analysis methods could affect the results of the studies from different laboratories (2,3,22). Thus, differences in the methods used to perform the comet assay and the influence of the study procedure are addressed to derive some conclusions regarding the use of the comet assay in future studies. In addition, different comet assay parameters have been used in studies by many researchers, which make comparison between the studies difficult. The accuracy and reliability of the results of a comet assay depend on the standardisation and implementation of these standards for all test steps, including the isolation of cells and the careful and precise evaluation of the results. In addition, the results of an analysis can vary with the person performing the analysis; thus, it is important that the same team performs a study and evaluates the results. Various studies have shown substantial differences among the amount of blood sample and washing buffers used in each study (23-27).

Our study utilised different conditions of the comet assay method that were applied in many areas of study by different laboratories. For this reason, we evaluated two different washing buffers and 7 different amounts of blood, thus generating 14 different test conditions. Two different slides were prepared for each sample. To eliminate inter-individual variability, all steps of the comet assay were conducted by the same investigator and analysis was performed by an automated system. Upon conclusion of our study, the ICCs among the 14 test conditions were high. Thus, perfect ICC was observed, especially between the two RPMI slides for each test condition.

We have shown that the blood samples and amounts of RPMI or PBS used in this experiment did not affect the results. All test conditions are comparable, and it is important that the test conditions have been examined using the same buffer, the same amount of blood sample, and the same analysis team throughout the process. Moreover, when the results of the PBS and RPMI buffers are compared, the ICC among test conditions with RPMI were higher than those with PBS. Thus, we suggest that, the comet assay should be performed using RPMI.

The evaluation of comet assay results using different parameters is problematic. There are many ways of measuring the level of DNA damage with the comet assay, including continuous measurements (computerised scoring in different units, such as percent DNA in the tail (%T), tail length and tail moment), categorical measurements (visual scoring in arbitrary units), and various descriptions of the distribution of the images (28) .

Automatic and visual photograph analyses are used while assessing the results, and visual analysis is applied in modern laboratories. However, we believe that relying on the use of automatic photograph analysis is more reliable and accurate. Many researchers have previously demonstrated that there is substantial variation among investigators scoring the same slides for the visual classification of comets (22,18). Briefly summarised, the factors contributing to inter-laboratory variability in comet assay results include the following: cell to cell variability, gel variability, slide variability, inter-individual variability, use of different image analysis systems, and/or visual scoring, and use of the comet parameters, e.g., olive tail moment, tail moment, tail intensity and tail (%) DNA(29).

To the best of our knowledge, this is the first study in which the comet assay was validated for 14 different test conditions and assessed according to 4 comet parameters. We have shown that the reliability and precision of the results are high when various amounts of PBS, RPMI, and blood samples are used. Therefore, our present study contributes to the literature by allowing future studies that use the comet assay to use less amounts of blood and RPMI or PBS, saving material and time.

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