

Approaches of Whole Blood Thawing for Genotoxicity Analysis in Rats

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Abstract

The comet assay is a sensitive method for detecting DNA damage and is widely used in isolated human lymphocytes. This test involves time-consuming experiments and the samples should immediately be processed after collection. Hininger et al. froze human whole blood samples to use for future analyzes and thawed them after four months. Herein, the objective of this study was to validate a protocol in rat whole blood samples following modified Hininger et al. protocol for future analyses of DNA damage. After rat blood collection, samples were randomly processed using four different tests: Test 1: Following Lima et al. protocol with freshly isolated rat lymphocytes, which are used as gold standard for comet assay. Test 2: This test as performed according to modified Hininger et al. protocol. Test 3: similar test to Hininger et al. freezing process, but with changes in thawing technique. Test 4: The fresh whole blood was used and considered as the control experiment. All the samples were prepared for DNA damage levels by comet assay. The results showed that the tests 1, 2, 3 and 4 showed no statistically significant differences ($p > 0.05$) among themselves, when analyzed using comet assay by software (tail intensity) or by visual score. Therefore, the tests with frozen whole blood were viable showing greater efficiency and reducing cost and time in laboratory practice. Thus, the present study also offers a new approach in experimental investigation, since most articles that address this issue is held in humans.

Keywords: Comet Assay; Whole blood; Rats; Frozen; Thawing; DNA damage

Introduction

External factors can influence the integrity of genetic material (DNA - deoxyribonucleic acid). Organism exposed to chemical, physical or biological agents are susceptible to DNA lesions. Many factors cause DNA damage, such as sunlight, environmental pollutants, stress, cigarette smoke exposure, reactive oxygen species (ROS), X-ray exposure and hyperglycemia. Several mechanisms involved in DNA damage are challenged, but the exact mechanism is not fully understood [1-3].

Cytogenetic markers such as chromosomal aberration assay, sister chromatid exchange assay, micronucleus test [4] and the comet assay [5] are used to evaluate the genotoxicity. The comet assay is a quick, simple, sensitive, reliable and fairly inexpensive way of measuring

DNA damage [6]. The genotoxic term is related to agents which change the DNA sequence and are considered to be toxic to the gene. Comet assay is not used for to detect genomic mutation but genomic injuries [7]. This method allows the detection of several classes of DNA damage as single and double strand breaks, alkali labile sites, incomplete repair sites and cross links [8]. Studies in humans showed that comet assay is widely used in isolated lymphocytes [9] because these provide a convenient and readily available source of human material. Furthermore, lymphocytes are used experimentally to evaluate potential toxic and cytoprotective effects in DNA damage and repair. The disadvantage of using the isolated human lymphocytes for comet assay involves time-consuming experiments and the samples should be immediately processed after collection. Hininger et al. [10] froze human whole blood samples to optimize the time and to use the samples for future analyses and thawed them after four months. These authors verified that this method was viable for DNA damage analysis.

For the reason that Hininger et al. [10] has been successful in standardizing an optimized protocol using human blood samples in the comet assay, our research group also intends to reproduce this protocol on whole blood samples from laboratory animals in order to reduce the time and costs involved in this technique. Herein, the objective of this study was to validate a protocol of rat whole blood samples following modified Hininger et al. [10] protocol for future analyses of DNA damage.

Materials and Methods

Animals

Three-month-old Wistar rats, weighing approximately 250 grams (g), were obtained from Center for Biological Research (CEMIB – Unicamp, Campinas, São Paulo State, Brazil). They were maintained in an experimental room under controlled conditions of temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$), and a 12-hour light/dark cycle.

Blood sampling

The different tests were used with isolated lymphocytes, frozen whole blood and fresh whole blood. The local Committee of Ethics in Animal Experimentation Unesp (CEEA – 952/2012) approved all experimental procedures presented in this study.

Samples freezing and thawing protocols

Adult male rats were anesthetized and whole blood was collected into EDTA-containing vacutainer tubes. Blood samples were randomly processed using four different tests ($n = 4$ animals/test) (Figure 1).

Test 1: The first test was performed with isolated lymphocytes due they are used as the gold standard for comet assay. This experiment following Lima et al. [11] protocol with rat freshly isolated lymphocytes. The lymphocytes were prepared with Ficoll-Paque[®] gradient and centrifuged with PBS (phosphate buffered saline). The samples were performed in duplicate for comet assay.

Test 2: This test was performed according to modified Hininger et al. [10] protocol. 500 μL whole blood was collected into EDTA-containing vacutainer tubes and stored in Eppendorf[®] tube with mixed of 400 μL of RPMI 1640 medium and 100 μL of dimethyl sulfoxide (DMSO). This test is similar of Hininger *et al.* (11) protocol but freezing step is different because the samples are gradually frozen in freezer -20°C for 24 hours and transferred to -80°C until analysis 4 months later. After quick thawing in water bath at 37°C , the blood samples were centrifuged at room temperature at 300x g for 5 minutes. The cell pellet was washed twice and re-suspended in Ca^{++} and Mg^{++} -free phosphate-

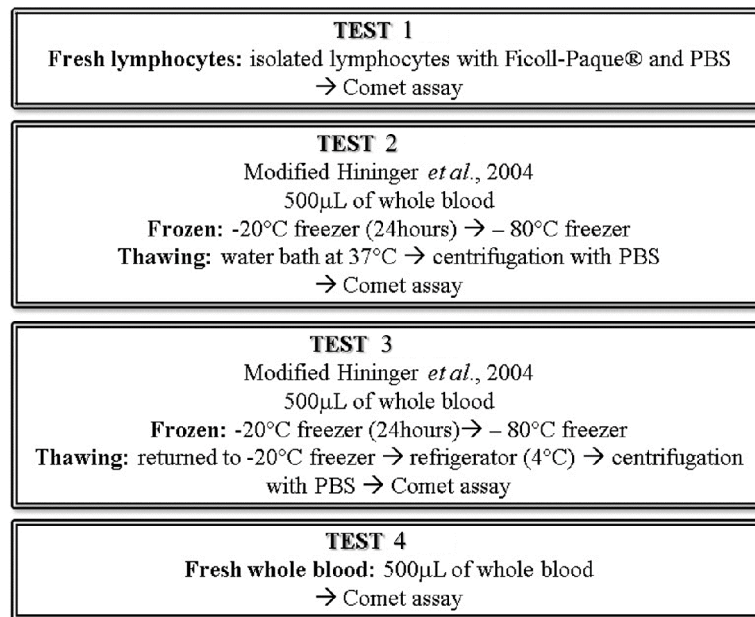


Figure 1: Samples freezing and thawing protocols.

buffered saline (PBS). Similarly to test 1, the samples were prepared for comet assay.

Test 3: The frozen was similar to test 2, but the thawing was different because the samples were removed of -80°C freezer and returned to -20°C freezer for 24 hours. Following, the samples were taken to refrigerator for 30 minutes. After this, all procedures about comet assay were similar to tests 1 and 2.

Test 4: In this test fresh whole blood was used and considered as the control experiment. 500µL whole blood was collected into EDTA-containing vacutainer tubes and the slides samples were similarly prepared to the other tests. The comet assay procedures were similar to tests 1, 2 and 3.

Comet assay

Chemicals: Chemical products used were purchased from the following suppliers: low and normal melting point agarose gels and EDTA from Invitrogen (Paisley, Scotland); sodium chloride, Tris, sodium hydroxide, N-lauroyl sarcosinate, Triton 100-X, dimethylsulphoxide (DMSO) and ethidium bromide (EtBr) from Sigma Chemicals Company (St. Louis, MO); phosphate-buffered saline (PBS) free calcium and magnesium from Gibco (Grand Island, NY).

Hydrogen peroxide (H₂O₂) treatment: Samples were treated with 400µM H₂O₂ for 5 minutes in ice following modified Blasiak et al. [12] protocol.

Slides preparation: Samples (20µl) were added to 120 µl of low melting point (LMP) agarose. The mixtures were layered onto slides precoated with normal melting point (NMP). The slides were immersed in lysis solution with 2.5M NaCl, 100 mM EDTA, 10mM Tris, 1% Triton X-100, 10% DMSO and 1% N-lauroyl-sarcosine overnight.

Electrophoresis: Then, the slides were washed in PBS for 5 minutes and immersed in a freshly prepared alkaline buffer (1mM EDTA, 300 mM NaOH (pH=13) in a horizontal electrophoresis tank, the protocol followed the general guidelines proposed by Tice et al. [13] modified [11]. After a 20 minutes DNA unwinding period, electrophoresis was conducted at 25 V and 300 mA for 30 minutes. Following 15 minutes neutralization with Tris (pH=7,5), the slides were fixed in absolute ethanol, and stored at 4°C.

Stain: Before analysis, the slides were stained with 50µl of 20µg/

ml ethidium bromide and scored.

Slide analysis: The slides were analyzed for visual score, through of 5 classes, from 0 (no tail) to 4 (almost all DNA in tail) [14], in total of 100 “nucleoids” (50 from each of two replicate slides per sample). Beyond visual score one hundred randomly selected cells (50 from each of two replicate slides) were evaluated from each sample and the tail intensity was determined. Tail Intensity, according to Comet Assay IV Image System (Perceptive Instruments, UK), is defined as ‘the sum of all intensity values in the tail region minus those which are part of the mirrored head region. The value is also expressed as a percentage of the total comet or cell intensity’ [15].

Statistical analysis

For comparison of DNA damage levels by visual score, non-parametric Kruskal-Wallis test following Dunn’s multiple comparison test was applied. For comparison of DNA damage levels in test 1, 2, 3 and 4 by software (tail intensity) was used Gamma distribution. Statistical significance was set at p<0.05.

Results

Table 1 presents DNA damage levels analyzed by visual score and software (tail intensity) among different thawing tests. There were no statistically significant differences in DNA damage levels among tests. The hydrogen peroxide (H₂O₂) treatment was performed for positive control.

Discussion

A study specifically designed to compare the DNA damage levels measured by the comet assay in freshly and frozen samples from animals have been not often reported. From this inquiry a study with laboratory animals was proposed. The use of whole blood using comet assay to measure DNA damage present on leukocytes is simpler and involves less handling of the cells, unlike what occurs when the isolation of lymphocytes is performed [16]. Unlike lymphocytes, few studies report on the use of whole blood to optimize the time in the experiments. Hininger et al. [10] used freshly and frozen blood samples of human and the samples were progressively frozen with culture medium RPMI 1640 and dimethyl sulfoxide (DMSO) and stored in a freezer at -80°C until four months. The thawing was performed in a water bath at 37°C and the samples were washed with PBS (phosphate buffered saline). There was no difference in the DNA damage levels between the freshly and

Table 1: DNA damage levels by visual score and software (tail intensity) analyses of whole blood and lymphocyte samples of adult male Wistar rats.

DNA damage	Test 1	Test 2	Test 3	Test 4
Visual score	1.12 ± 0.93	1.16 ± 0.58	1.22 ± 0.56	1.20 ± 0.40
Tail intensity (%)	33.32 ± 16.46	33.65 ± 16.20	36.78 ± 18.82	34.54 ± 17.15

Test 1- freshly isolated lymphocytes; **Test 2-** frozen whole blood (4 months) and thawing in water bath at 37°C; **Test 3-** frozen whole blood (4 months) and gradually thawing; and **Test 4-** freshly whole blood.

Data are expressed as mean ± standard deviation (SD)

p > 0.05 – no statistically significant difference

frozen whole blood samples analyzing by comet assay. It is relevant to discuss the time factor and the costs generated in the bench tests for genotoxicity analysis to minimize the time for sample processing. According to our results, the tests 1, 2, 3 and 4 showed no differences with respect to the DNA damage levels, showing that the frozen whole blood samples were not affected by freezing and/or thawing. Furthermore, the genotoxicity analysis by visual score or employing the comet assay IV software to obtain data of tail intensity did not differ between both.

There is extensive discussion in the literature that the red blood cells and platelets do not contribute to the DNA damage analysis by comet assay because these cells present no nuclei. Narayanan et al. [17] verified the influence of red blood cells in *in vitro* whole blood samples for break single strands of DNA in human lymphocytes. DNA damage increased as the lymphocytes were analyzed in the presence of red blood cells compared with isolated lymphocytes. According to Chuang & Hu [18], the red blood cells do not interfere in the damage levels using the comet assay. These authors explain that the red cells are lysed in two steps of the assay, the incubation time in lysis solution and in electrophoresis.

Al-Salmani et al. [19] investigated the applicability of comet assay to a whole blood, varying the volume of blood collected and the effects of various storage temperatures. Blood was processed immediately for comet assay or frozen, and the storage temperature was 4°, -20°C, or -80°C, with or without 10% DMSO, in different periods. These authors were able to detect a nucleobase damage in the frozen samples, similar to that seen for the samples processed immediately without freezing. Another study using leucocytes in whole blood (whether fresh or frozen) for comet assay demonstrated that these cells are applicable and present great potential in biomonitoring for DNA damage measurement with the comet assay [20].

In addition to the DNA damage analysis, it is important to note the costs in each test performed in our study. The test 2 (modified Hininger et al. protocol [10]) represent new possibilities for sample collection and storage aiming further analysis because it excludes the step for lymphocyte isolation. This fact reduces the cost of reagents and other materials. The test 3 was also considered feasible for the study because the samples were thawed using similar equipment to those used for freezing. However, the methodology that uses freshly whole blood and lymphocyte isolation presents a disadvantage because it must be prepared immediately after blood collection. After extensive analysis of the articles that mention the use of freshly or cryopreserved whole blood or lymphocytes, it was found that there are many differences in the intra- and interlaboratory results regarding the genotoxicity analysis using comet assay. This is due to several factors such as agarose concentration, alkaline incubation time, voltage, current and time of electrophoresis and also quantity of electrophoresis buffer, which can interfere with the current, since water is conductive energy [21].

Conclusion

In conclusion, according to our results the tests performed with frozen whole blood were viable because there was reduction of the blood volume used as compared with that used for lymphocyte isolation; it was not necessary the lymphocyte isolation process,

eliminating many hours of technique and showing better efficiency and reduced cost in laboratory practice. Thus, the present study also offers a new approach in experimental investigation, since most articles that address this issue is held in humans.

Conflicts of Interest

The authors have no competing interests to declare.

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